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The gene encoding the neu/erbB-2/HER-2 receptor tyrosine kinase is one of a small number of genes known to be altered in human breast and ovarian carcinoma. It is amplified and/or over expressed in approximately one-fourth of these cancers. With its probable role in etiology, and expression at the cell surface, Neu has great potential as a prognostic indicator and therapeutic target. Efforts to exploit this receptor in the clinical arena have been impeded by the failure to consider regulation of Neu by hormones and co-receptors. This has been complicated by the fact that at least seven different peptide hormones, epidermal growth factor (EGF), transforming growth factor-alpha, amphiregulin, betacellulin, heparin-binding EGF-like growth factor, epiregulin, and the diverse neuregulins are all able to activate neu. They work by binding to the co-receptors EGF receptor, erbB-3 and erB-4, which then dimerize with neu. Work by this laboratory has shown that each one of these factors activates a different constellation of receptors, leading to a great diversity of possible response. Moreover, the coupling of cellular responses to each factor is determined by the subset of receptors expressed in each tissue. Thus in order to interpret the function of any one of the factors or receptors, including neu, it is essential to consider the influences provided by the entire set.

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INTRODUCTION

Breast cancer is one of the most important cancers afflicting women and presents challenging treatment decisions. After p53 alterations, the most frequent change in an identified gene is amplification and/or overexpression of the *neu/erbB-2/HER-2* gene, which occurs in up to one-third of breast cancers. The gene product (denoted p185 or non-italicized neu), is a receptor tyrosine kinase (RTK) [1, 2, 3]. This gene was originally discovered in mutant form in chemically-induced rat nervous system tumors and is now known to be a member of the Type 1, or epidermal growth factor (EGF) receptor gene (*erbB*) family. The family includes four receptors, which will be referred towill be referred to herein as the EGFR, neu, erbB-3, and erbB-4. (See our review, [4]). Small-scale screens for *neu* alterations in human tumors led to discovery of changes in a number of adenocarcinomas including breast [5], ovarian, gastric, bladder, lung, and colon. Two influential studies showed that *neu*, and not a panel of other oncogenes is amplified in breast and ovarian carcinomas and that this amplification correlates with RNA and p185 overexpression [6, 7]. Numerous studies of *neu* in mammary carcinoma have led to the following conclusions [4]:

1.*neu* is amplified in 20-30% of mammary carcinomas, with the frequency of amplification higher in tumors from patients with affected lymph nodes [8, 9, 10, 11, 12, 13, 14, 15, 16]. The gene amplification suggests that there is a selection in the tumors for *neu* overexpression. (It cannot be absolutely ruled out that neighboring genes are selected for amplification.)

2.Amplification correlates well with concomitant RNA and p185 expression [6, 7]. An additional 5% of specimens overexpress the receptor without obvious changes in gene structure or copy number [5, 11, 15, 17].

3.There is no evidence for structural mutations in p185 in human tumors. This negative result is weak owing to the high copy number of genes and the large size of the

mRNA. Recent work in the mouse transgenic system suggests that this issue should be reconsidered [18].

4.Amplification and/or p185 overexpression can be found in all grades and stages of carcinomas, but not hyperplasia or dysplasia. It is found more frequently in ductal carcinoma in situ (DCIS) than in infiltrating ductal carcinoma (IDC)[14, 16, 19, 20].

5.Amplification and/or p185 overexpression is associated with poor prognosis, especially in node-positive patients. However the extent of this association and independence from other prognostic markers varies greatly among different studies (reviewed, [4]).

Taken together, these data indicate that *neu* amplification and overexpression play a major role in mammary carcinogenesis. This is consistent with findings in model systems: i) the mutated rat *neu* oncogene is as potent as any including *ras* in tissue culture systems. ii)In contrast to other growth factor receptors including the EGFR, overexpression of p185 in the absence of ligand is sufficient to transform cells [21, 22]. iii)Transgenic mice harboring a mutationally activated *neu* oncogene develop multi-focal mammary carcinoma when expressed under control of a murine mammary tumor virus (MMTV) promoter, which confers high level expression in mammary gland and a few other tissues (not found in all studies [23, 24]. iv)Perhaps most compelling is the fact that transgenic mice carrying a structurally normal *neu* gene driven by the MMTV promoter develop metastatic mammary carcinomas [25]. This is noteworthy because it reconstructs what appears to be occurring in human cancer: overexpression of normal p185 in mammary tissue.

Since p185^{neu} is a cell surface protein that seems to play a causal role in mammary carcinogenesis, it is under intensive investigation as a therapeutic target [4, 26]. Phase II clinical trials, in which patients were infused with anti-neu antibody 4D5 have been completed with a roughly 15% response rate, and represent the vanguard for expanded therapeutic trials targetting this receptor (J. Baselga, personal communication). In spite of

the findings linking *neu* to mammary carcinoma, and despite the fact that patients are already being exposed to neu antagonists, little is known about the function of neu either in the organism, or in breast cancer.

The physiological function of p185*neu*, like any hormone receptor, can only be understood in the context of the hormones that regulate it. The EGFR is activated by binding of at least six different peptide hormones, EGF, TGF-α, amphiregulin (AR), betacellulin (βC), epiregulin [27](epi) and heparin-binding EGF-like growth factor (Hb-EGF)[28, 29, 30, 31]. p185, by itself, cannot bind or be activated by these hormones (epi has not been tested). We discovered that EGF and TGF-α, which do not bind to p185, activate p185 Tyr phosphorylation and stimulate p185-associated kinase activity [2, 32]. This phenomenon, now termed **transmodulation**, is dependent upon the co-expression of the EGF receptor with p185 [33, 34, 35, 36]. It probably occurs at least in part through formation of receptor heterodimers [37, 38]. Transmodulation of neu by the EGFR is biologically relevant since it works with EGF, TGF-α, betacellulin (see below) and AR, stoichiometrically activates p185 [32], permits association of substrates [39], and correlates with *in vivo* synergy in transforming ability of these two receptors [40]. Thus wherever the two receptors are co-expressed, EGFr agonists activate *neu* . In cell lines that express both receptors, EGF-regulated neu signaling is at least as important as signaling by the EGFR [41]. Since p185 and the EGFR have distinguishable signaling activities [42], this means that regulation of neu production provides a means to alter the signal coupled to EGF.

The transmodulation of neu by the EGFR is a prototype for other interactions within the Type 1 receptor family discovered more recently. Ignorance of these interactions has confused many groups studying the EGFR and resulted in a rather muddy literature which is just now being rationalized [4, 43]. For example, several laboratories independently identified an activity termed Heregulin, neu diffentiation factor, gp30, p75, neuregulin, ARIA, and Glial Cell Growth Factor (GGF) [44, 45, 46, 47, 48, 49, 50, 51], a family of related proteins evidently produced by alternate splicing[50](They

will be referred to collectively here as NRG, for the composite name neuregulin, or as NDF). At first the NRGs seemed to be neu ligands since they activate p185 tyrosine phosphorylation in the absence of the EGFR and could be cross-linked to neu [45, 49]. However, it is now known that NRGs bind to both erbB-3 and erbB-4 which can then activate neu by transmodulation [52, 53]. A further complication of this receptor system is that erbB-3 lacks robust kinase activity, and itself requires a second receptor for activity [54].

Additional candidates for neu ligands have been identified but not yet expressed in recombinant form and tested for activity [55, 56]. Nonetheless, the independent purification of NRGs by three different laboratories seeking the *neu* ligand suggests that in mammary epithelia the significant inputs to *neu* may come through transmodulation: transmodulating agonists TGF-α, AR, and NDFs are often produced in mammary tissue or cell lines [57, 58, 59] as are the cooperating receptors. Even if these hormones are uniquely responsible for neu activation in mammary tissue, the biological complexities may be enormous. TGF-α and AR, although both EGFR agonists, have somewhat different biological activities [28]. NRGs at first seemed to have radically different activities than EGF agonists since they promote differentiation in some cell lines [49, 60] (but not others [45, 50]), but this has still not been verified in tissue[4].

In summary, *neu* amplification and overexpression is likely to play a significant role in carcinomas where it occurs. However, the presence of activating mutations and agonistic peptide hormones will regulate *neu* function much more strongly than abundance. The Type 1 RTKs comprise a network in which the signaling potential of each receptor is conditioned not only by the presence of hormones, but is further regulated by the co-expression of related RTKs. The long-term focus of this grant is to define the capabilities of the erbBfamily receptor network: the spectrum of hormones that activate each receptor and receptor combination, and the differences among signalling pathways governed by these receptor systems. These objectives include:

Aim 1: Signalling of individual receptors and receptor combinations will be compared by investigating receptor phosphoryaltions and substrate phosphorylations to determine how receptor interactions modulate signalling specificity.

Aim 2: Functions of NRG ecto- and endo-domains will be analyzed.

Aim 3: Biological activity of NRGs and NRG/TGF-α combinations will be determined in tissue culture.

BODY

ErbB Receptor Signaling Network

The foremost objective for year 1 (Tasks 1a and 1c) was to determine the ability of each receptor and receptor combination to respond to each of the EGF family agonists. This was done by using stable cell lines produced in year 1 that express erbB, erbB-2, erbB-3, and erbB-4 singly and in all pairwise combinations. In year 1 we completed most of the analysis of the ligands EGF, TGF-α, AR, Betacellulin, NRG, and HbEGF [61]. This work was completed in year 2, with publication of 2 additional papers [62, 63]. In year 2 we undertook analysis of the remaining EGF-related factor described in the original proposal, Cripto-1. Since the goal of this work is a comprehensive analysis of EGF-related factors, we also felt obligated to investigate two factors discovered more recently than the original proposal: epiregulin [27], and NRG-2. Work on Cripto-1, NRG-2, and epiregulin is now complete, and has resulted in two publications[64,65] and one manuscript submitted[66]. Cripto-1 doesn't activate any of the receptors, NRG-2 binds to ErbB-3 and ErbB4, and Epiregulin strongly activates the EGF receptor, but weakly activates ErbB4. Responses of ErbB4 to Betacellulin and to Epiregulin is greatly enhanced in the presence of ErbB-2. This work marks the end of virtually all of Task 1.

Aim 1, Task 2. Mammary Cell Lines. There is mounting evidence that while activation of either EGFR or erbB-2 stimulates mammary cell proliferation and promotes tumorigenesis, increased erbB4 signaling may inhibit proliferation or tumorigenesis by stimulating differentiation. Ectopic treatment of breast tumor cell lines with NRG inhibits their growth and stimulates milk protein synthesis. Moreover, erbB4 overexpression in human mammary tumor samples correlates with markers for a more favorable prognosis, suggesting that erbB4 signaling may inhibit tumorigenicity [Bacus, *et al.*, 1996].

As discussed last year, Task 2 turned out to be not feasible, since MCF-10A cells do not tolerate expression of ErbB-4. We are continuing to approach this problem using the mouse mammary cell line HC11. HC11 cells are a Balb/c mammary cell line cultured

from a mid-pregnant female that can be induced to differentiate and activate milk protein synthesis (β-casein) in the presence of dexamethasone, insulin and PRL (DIP). In order to acquired DIP responsiveness, HC11 cells must first become "competent" through confluent incubation in the presence of EGF. However, EGF actually suppresses induction of β-casein by DIP. This latter response is mediated by Stat5. In contrast to EGF, mutationally activated ErbB-2 induces competence, but has no inhibitory effect on β-casein. Finally, NRG resembles EGF in inducing competency, and inhibiting β-casein production, and inducing production of a 22kd milk protein in the presence of DIP. These EGF-like properties and failure to induce full differentiation by NRG contrast with results of pellet implant experiments. Although there are numerous differences between the in vitro and in vivo situations, there may be a simple explanation. We have found that HC11 cells express EGFR, ErbB-2, and ErbB-3, but do not produce ErbB-4 mRNA detectable by RNase protection. We hypothesize that introduction of ErbB-4 into these cells will convert the NRG response to a differentiation response, and are currently testing these idea as an alternative approach to Task 2.

II.NRG Intracellular Domain

The cytoplasmic domain of NRG family EGF-related growth factors is unusual in showing extraordinary diversity of regulation by splicing, and in that some forms have unusually long cytoplasmic tails of unknown function (over 400 amino acids). We hypothesize that these tails are likely to themselves transmit signals so that binding of NRG ecto-domains to the cognate receptors results in bidirectional signaling.

Task 3b. The major focus of work on the NRG intracellular domain has been to develop physical and two-hybrids screens to identify binding partners. In order to screen for a broad range of potential interactors, we are using a cDNA library made from a 12-13 day whole murine fetus. We have produced two baits, *GAL4*-BD- exon 11, and -exons 11+12+13, and verified expression by immunoblotting with anti-Gal4. With 4.2 X 10^6 clones screened), 6 positives have completed the the screen. The second bait has yielded

75 positives in the first round only, and we are currently working through the remainder of this screen. Once these two screens are completed, we will identify one or two best-guess candidates for further analysis, and use reconstruction experiments and other means to determine their validity. We anticipate that this work will identify truly novel functions for these intracellular domain peptides.

Glutathione-S-transferase (GST)-fusion proteins. A complementary method for identification of interacting proteins is affinity purification. This has the advantage of using full-length cellular proteins, that are expressed in their native environment, but probably requires stabler interactions than two-hybrids screening. We are using glutathione agarose affinity purification to identify proteins that bind specifically to GST-fusions of NRG cytoplasmic segments. Once positives are identified, this approach can be used for purification for direct sequencing, to verify positives identified in two-hybrids screens, and/or to verify positives from solid phase screening (see below).

GST fusion proteins have been produced with GST-coding sequences fused to the three intracellular exons. These fusion proteins express well, and are being used currently in pilot "pull-down" and Far-Western experiments to identify potential interactors. In preliminary experiments, the three GST-fusions isolate different bands in pull-down experiments with 35S-Met/Cys-labeled extracts from a mammary carcinoma cell line. This approach does not lead directly to molecular clones, but may identify candidates with deduced identities. For Far-Western experiments, the fusion proteins will be labeled in vitro by phosphorylation with cAMP-dependent protein kinase catalytic subunit, since the GST-tag (in pGEX2TK) also includes a phosphorylation site. This probe will be used to identify specific bands in blots. Ideally, GST-affinity purification will identify the same proteins as revealed by Far-western analysis, helping to confirm both results. If the results are clean, the next step will be to use the same probes in screens of cDNA expression libraries.

CONCLUSIONS

In year 3, we finally completed our comprehensive analysis of interactions of ErbB family receptors with their ligands[67]. This work, provides a resource that will benefit all investigators working in the EGF receptor family. This work has resulted in four new published or submitted papers. The definition of the abilities of individual EGF-related hormones to regulate specific receptors and receptor combinations is absolutely essential for understanding the biology of any of these hormones and their receptors and is a significant contribution of this work. This "wiring diagram" will benefit all investigators working in the system and is a unique contribution of our laboratory. Moreover, since we have determined that ErbB-4 may antagonize mammary proliferation, our ability to predict which hormones directly activate ErbB-4 may have clinical significance.

We are now investigating the use of a rodent model, HC11 cells, for pinpointing the connection between ErbB family receptors and mammary epithelial response. This will provide an important in vitro system that will be integrated with transgenic work we are performing to address the role of these receptors in normal mammary epithelium. Results from these model systems will be important in explaining how normal cellular responses coupled to these receptors go awry in breast cancer.

Finally, we have completed most of the ground-work for elucidating the function of the NRG tail, and nearly completed the two-hybrids screens. This work will lead to identification of new signaling functions for these ligands, that will be important in understanding their role in mammary tissue and other locales in which NRGs function.

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APPENDIX

new publications from the grant from the current project period

Kannan, S., DeSantis, M., Lohmeyer, M., Riese II, D.J., Smith, G.H., Hynes, N., Seno, M., Brandt, R., Bianco, C., Persico, G., Kenney, N., Normanno, N., Martinez-Lacaci, I., Ciardello, F., **Stern**, D.F., Gullick, W.J., Salomon, D.S. 1997. Cripto enhances the tyrosine phosphorylation of Shc and activates MAP kinase in mammary epithelial cells. J. Biol. Chem.272: 3330-3335.

Chang, H., Riese II, D.J., Gilbert, W., **Stern**, D.F., and U.J. McMahan. 1997. Ligands for ErbB family receptors encoded by a newly characterized neuregulin-like gene. Nature: 387: 509-512.

Riese II, D.J., and **Stern**, D.F. Specificity Within the EGF Family/ErbB Receptor Family Signaling Network. 1998. BioEssays *in press.*

Riese, II, D.J., Komurasaki, T., Plowman, G.D., and **Stern**, D.F. Activation of ErbB4 by the bifunctional EGF family hormone epiregulin is regulated by ErbB2. *submitted for publication.*

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Cripto Enhances the Tyrosine Phosphorylation of Shc and Activates Mitogen-activated Protein Kinase (MAPK) in Mammary Epithelial Cells*

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Cripto-1 (CR-1), a recently discovered protein of the epidermal growth factor (EGF) family, was found to interact with a high affinity, saturable binding site(s) on HC-11 mouse mammary epithelial cells and on several different human breast cancer cell lines. This receptor exhibits specificity for CR-1, since other EGF-related peptides including EGF, transforming growth factor α, heparin-binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin, or heregulin β1 that bind to either the EGF receptor or to other type 1 receptor tyrosine kinases such as *erb* B-3 or *erb* B-4 fail to compete for binding. Conversely, CR-1 was found not to directly bind to or to activate the tyrosine kinases associated with the EGFR, *erb* B-2, *erb* B-3, or *erb* B-4 either alone or in various pairwise combinations which have been ectopically expressed in Ba/F3 mouse pro-B lymphocyte cells. However, exogenous CR-1 could induce an increase in the tyrosine phosphorylation of 185- and 120-kDa proteins and a rapid (within 3–5 min) increase in the tyrosine phosphorylation of the SH2-containing adaptor proteins p66, p52, and p46 Shc in mouse mammary HC-11 epithelial cells and in human MDA-MB-453 and SKBr-3 breast cancer cells. CR-1 was also found to promote an increase in the association of the adaptor Grb2-guanine nucleotide exchange factor-mouse *son of sevenless* (mSOS) signaling complex with tyrosine-phosphorylated Shc in HC-11 cells. Finally, CR-1 was able to increase $p42^{erk-2}$ mitogen-activated protein kinase (MAPK) activity in HC-11 cells within 5–10 min of treatment. These data demonstrate that CR-1 can function through a receptor which activates intracellular components in the *ras/raf*/MEK/MAPK pathway.

The human cripto-1 (CR-1)[1] gene (also known as teratocarcinoma-derived growth factor-1 (TDGF-1)) is located on chromosome 3p21·3 and codes for a 28–36-kDa glycoprotein of 188 amino acids. CR-1 possesses an epidermal growth factor (EGF)-like consensus sequence that contains six cysteine residues in a region of approximately 37 amino acids (1). However, unlike other peptides within this family of growth factors that have a three-looped EGF-motif (designated A, B, and C) which are formed by three intramolecular disulfide bonds, the EGF-like repeat in CR-1 lacks an A loop and the B loop is truncated (2, 3). In addition, unlike most growth factors in the EGF family, the human CR-1 protein lacks a conventional hydrophobic signal peptide and a classical hydrophobic transmembrane domain (2). Nevertheless, recombinant human CR-1 protein is secreted when transiently expressed in Chinese hamster ovary cells (3). Refolded CR-1 peptides that correspond to the EGF-like repeat within the human CR-1 protein are mitogenic for nontransformed and malignant human and mouse mammary epithelial cells (3). Human CR-1 can also function as a dominant transforming gene *in vitro* in mouse NIH-3T3 fibroblasts and in mouse NOG-8 mammary epithelial cells (1, 4). CR-1 mRNA and immunoreactive CR-1 protein are differentially expressed in several human breast cancer cell lines, in approximately 80% of primary human infiltrating breast tumors and in 50% of ductal carcinomas *in situ* (5, 6).

Peptide growth factors that are members of the EGF family include transforming growth factor α (TGFα), amphiregulin (AR), heparin-binding EGF-like growth factor, betacellulin (BTC), epiregulin, and the neuregulin subfamily that consists

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[1] The abbreviations used are: CR-1, cripto-1; EGF, epidermal growth factor; TGFα, transforming growth factor α; AR, amphiregulin; HRG, heregulin; BTC, betacellulin; EGFR, EGF receptor; mSOS, mouse *Son of Sevenless*, gunanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; *erk*, extracellular signal-regulated protein kinase; MEK, mitogen-activated *erk*-activating kinase; MBP, myelin basic protein; Shc, *Src* homologous and collagen protein; Grb2, growth factor receptor-bound protein; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

of various isoforms of α and β heregulin (HRG), gilial cell growth factors, and acetycholine receptor inducing activity (7–10). The different neuregulins are derived by alternative splicing from a single gene. Peptides in the EGF family bind to and activate members of the *erb* B family of type 1 receptor tyrosine kinases. EGF, TGFα, AR, heparin-binding EGF-like growth factor, and epiregulin bind exclusively to the epidermal growth factor receptor (*erb* B/EGFR), whereas the neuregulin subfamily of peptides bind to c-*erb* B-3/HER-3 or c-*erb* B-4/HER-4 that can then heterodimerize and activate c-*erb* B-2/HER-2 following transphosphorylation (11–15). BTC can bind to either the EGFR or c-*erb* B-4. Since ligand-dependent activation of the EGF receptor can also lead to heterodimerization with c-*erb* B-2, c-*erb* B-3, or c-*erb* B-4, this suggests that different pairs of heterodimers within the type 1 receptor tyrosine kinase family may contribute to the array of responses to various EGF-like ligands in a cell-specific and possibly ligand-specific manner by the recruitment of different combinations of intracellular signaling proteins (14). This apparent redundancy of different ligands and receptors may therefore contribute to signal diversification and amplification. One major pathway that is activated by a several different EGF-like ligands through these type 1 receptor tyrosine kinases is the *ras/raf*/mitogen-activated protein kinase (MAPK) signal transduction pathway (16).

The HC-11 mouse mammary epithelial cell line is a clonal isolate originally derived from the COMMA-1D cell line, which was established from a midpregnant Balb/c mouse mammary gland (17). These cells express a number of mammary epithelial markers such as β-casein and express several distinct type 1 receptor tyrosine kinases, including the EGFR, c-*erb* B-2, and c-*erb* B-3 (17–20). In this context, HC-11 cells have been useful as an *in vitro* model system in which to define the intracellular signaling pathways that are engaged by EGF-related peptides that utilize these type 1 receptor tyrosine kinases and are involved in regulating proliferation and differentiation in mammary epithelial cells (19, 20). In the present study, we have attempted to define the intracellular signaling pathway that might be activated in HC-11 cells and in several different human breast cancer cell lines by a 47-mer refolded CR-1 peptide that corresponds in sequence to the EGF-like repeat of the human CR-1 protein (3). The CR-1 related peptide was found to bind to a unique specific, high-affinity receptor that is not the EGFR, *erb* B-2, *erb* B-3, or *erb* B-4. CR-1 was also found to enhance the tyrosine phosphorylation of the SH2-adaptor protein, Shc, and to promote the association of Grb2-mSOS intracellular signaling complex with tyrosine-phosphorylated Shc. These events were subsequently related to the downstream activation of the p42$^{erk\text{-}2}$ MAPK isoform.

MATERIALS AND METHODS

Cell Culture and Growth—HC-11 mouse mammary epithelial cells were routinely grown in RPMI 1640 medium containing 8% heat-inactivated fetal bovine serum, 5 μg/ml bovine insulin (Sigma), and 10 ng/ml EGF (Collaborative Research, Watham, MA). Human breast cancer cell lines, MCF-7, ZR-75-1, T-47D, MDA-MB-231, MDA-MB-453, and SKBr-3 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and insulin (10 μg/ml) as described previously (21). The Ba/F3 mouse pro-B lymphocyte cell line and clonal derivatives that express individual or pairs of type 1 receptor tyrosine kinases were generated as described previously (22, 23). Cells were treated with different concentrations of either refolded p47 CR-1 peptide that corresponds in sequence to the EGF-like motif in human CR-1 (3), recombinant human TGFα (Bachem), recombinant human AR, recombinant human HB-EGF, recombinant human BTC (R & D Systems, Inc.), recombinant mouse epiregulin (generously supplied by Toshi Komuraski, Taisho Pharmaceutical Co., Saitama, Japan), or recombinant human HRG $\beta1_{177-244}$ (generously supplied by Mark Sliwkowski, Genentech, Inc., South San Francisco, CA).

Immunoprecipitation and Western Blot Analysis—Cells were grown until they were subconfluent and then propagated in serum-free medium containing human transferrin (10 μg/ml) and type 1V Pedersen fetuin (1 mg/ml) for 48 h. Cells were treated in serum-free medium with different concentrations of p47 CR-1 for various times and then lysed in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM $MgCl_2$, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 20 mM sodium fluoride. In some cases the clarified protein lysates were either immunoprecipitated (0.5 mg/sample) with 2 μg of a rabbit anti-Shc antibody (Transduction Laboratories, Lexington, KY) or with 2 μg of anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.). Crude protein lysates (25 μg/sample) or immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, blocked with 2% dry milk in Tris-buffered saline with 0.05% Tween 20, and incubated with a 1:1000 dilution of anti-Shc, anti-Grb-2, or anti-mSOS monoclonal antibodies (Transduction Laboratories) or a 1:2000 dilution of the anti-phosphotyrosine monoclonal antibodies 4G10 and/or PY-20 (ICN Pharmaceuticals, Inc., Costa Mesa, CA). The bound mouse monoclonal antibodies were detected using a 1:5000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham). Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham). *erb* B receptors were immunoprecipitated from recombinant Ba/F3 clonal derivatives using monospecific antisera as described previously (22).

MAPK Assays—A rabbit anti-MAPK antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at a 1:1000 dilution that recognizes both extracellular signal-regulated protein kinase *erk-1* and *erk-2* MAPK was used to detect activation of immunoreactive MAPK proteins by means of band shift following SDS-PAGE of crude cellular lysates as described previously (24). Bands were detected by the colorimetric NBT/BCIP system (Kirkegaard Perry Labs, Gaithersburg, MD). Alternatively, cells were lysed in buffer containing 1% Nonidet P-40, 0.5% deoxycholate, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM sodium orthovanadate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Lysates (100 μg/sample) were immunoprecipitated with an anti-*erk-1* rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After 2 h, the immunoprecipitates were washed twice with lysis buffer followed by a final wash in kinase buffer containing 30 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. After the final wash, the immunoprecipitates were suspended in 30 μl of kinase buffer containing 5 μg of myelin basic protein (MBP) and 5 μCi of [γ-^{32}P]ATP (1000 Ci/mmol, Amersham) and incubated for 30 min at 37 °C. The reaction was stopped with 2 × SDS sample buffer. The samples were then run on a 10% Tricine gel followed by autoradiography. The MBP bands were quantified with a densitometer (Molecular Dynamics) (19).

Ligand Binding Assays—^{125}I-p47 CR-1 peptide was prepared using IODO-GEN (Pierce Chemical Co.) in which 5 μg of peptide was labeled with 1 mCi of Na^{125}I to a specific activity of 5–10 μCi/μg. Monolayers of HC-11 cells or human breast cancer cells in 12-well cluster dishes were washed in serum-free RPMI 1640 or Dulbecco's modified Eagle's medium containing 20 mM HEPES (pH 7.4) and 0.1% bovine serum albumin and treated in the same medium. Cells were incubated at 23 °C for 2 h in 1 ml of binding buffer containing 3×10^5 cpm of ^{125}I-p47 CR-1 in the absence or presence of increasing concentrations of unlabeled p47 CR-1 or with other EGF-related peptides. Cells were washed twice with phosphate-buffered saline and then solubilized in 1 ml of buffer containing 10 mM Tris-HCl (pH 8.0) and 0.5% SDS and counted in a γ-counter. The binding data were analyzed using the Ligand program to determine the K_d value as described (25).

RESULTS

Binding of CR-1 to HC-11 Mouse Mammary Epithelial Cells and Human Breast Cancer Cells—A 47-mer CR-1 refolded peptide that contains the EGF-like motif of human CR-1 is able to stimulate the proliferation and to inhibit β-casein expression in mouse HC-11 mammary epithelial cells.[2] To determine if a high-affinity, specific binding site is expressed on HC-11 mammary epithelial cells that might mediate the biological effects of CR-1, HC-11 cells were incubated with ^{125}I-p47 CR-1 in the absence or presence of increasing concentrations of unlabeled p47 CR-1. As illustrated in Fig. 1A, there is specific binding of the ^{125}I-p47 CR-1 peptide to HC-11 cells with a K_d of approxi-

[2] S. Kannan, manuscript in preparation.

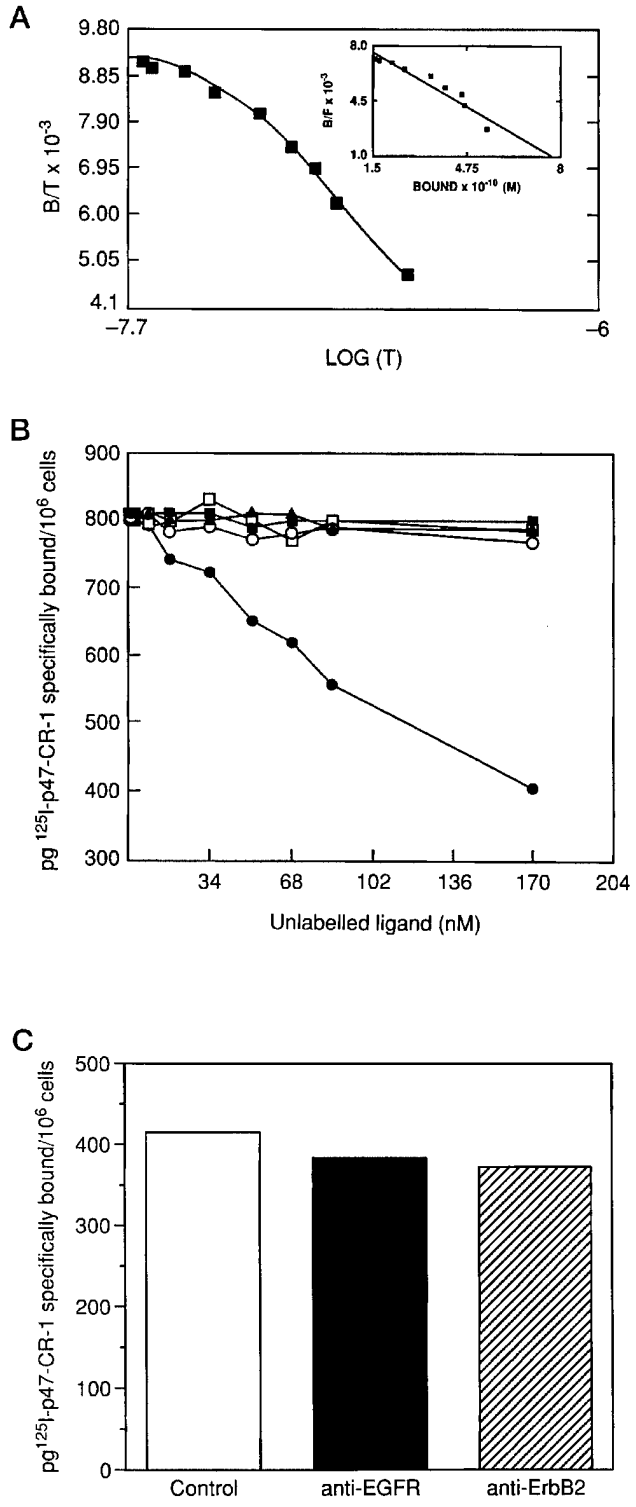

FIG. 1. **Binding and competition curve of ^{125}I-p47 CR-1.** Binding of ^{125}I-p47 CR-1 to HC-11 cells (*A*); *insert*, Scatchard plot of binding isotherm. *B*, competition of specific binding of ^{125}I-p47 CR-1 to HC-11 cells by various concentrations of EGF (○), AR (□), TGFα (■), HRGβ1 (▲), or p47 CR-1 (●). *C*, MDA-MB-231 human breast cancer cells were treated with 25 μg/ml anti-EGFR monoclonal antibody or 25 μg/ml anti-*erb* B-2 TAb 250 monoclonal antibody and specific binding of ^{125}I-p47 CR-1 (10 ng/ml) was determined.

mately 96 nM (Fig. 1*A*, *inset*). Various concentrations of EGF, TGFα, AR, or HRGβ1 were unable to compete for binding with ^{125}I-p47 CR-1 on HC-11 cells (Fig. 1*B*). Likewise, heparin-binding EGF-like growth factor, epiregulin, or BTC were ineffective in blocking the binding of the ^{125}I-p47 CR-1 peptide (data not shown). This potential receptor is not unique to HC-11 mouse mammary epithelial cells since ^{125}I-p47 CR-1 also binds specifically and with high affinity to several estrogen receptor positive (MCF-7, T47-D, and ZR-75–1) and to estrogen receptor negative (MDA-MB-231, MDA-MB-453 and SKBr-3) human breast cancer cell lines that exhibits comparable specificity for CR-1 binding as in mouse HC-11 cells (Table I). Moreover, blocking the EGFR receptor on MDA-MB-231 human breast cancer cells with 25 μg/ml of the 528 anti-EGFR monoclonal blocking antibody which is sufficient to impede ^{125}I-EGF binding (26) does not lead to any change in the specific binding of ^{125}I-p47 CR-1 to these cells (Fig. 1*C*), confirming the observation that CR-1 does not directly bind to the EGFR (3). In addition, the binding of ^{125}I-p47 CR-1 peptide to MDA-MB-231 cells or SKBr-3 cells (data not shown) is unaffected by the anti-*erb* B-2 TAb 250 blocking monoclonal antibody.

TABLE I
Characteristics of CR-1 binding to mouse and human mammary epithelial cells

Data were obtained from appropriate Scatchard plots using various concentrations of unlabeled p47 CR-1 with ^{125}I-p47 CR-1.

Cell line	nM	Sites/cell
HC-11	96	3.9×10^5
MDA-MB-453	80	4.4×10^5
MDA-MB-231	33	1.8×10^5
SKBr 3	78	4.5×10^5
ZR-75–1	61	1.1×10^5
T47-D	50	1.4×10^5
MCF-7	26	5.3×10^4

CR-1 Fails to Directly Activate erb B Family Receptor Tyrosine Phosphorylation in Recombinant Ba/F3 Cells—Parental Ba/F3 mouse pro-B lymphocytes express low levels of endogenous *erb* B-3 but do not express EGFR, *erb* B-2, or *erb* B-4. Ba/F3 clones have been genetically engineered to ectopically express either EGFR, *erb* B-2, *erb* B-3, or *erb* B-4 type 1 receptor tyrosine kinases either alone or in different pairwise combinations (22, 23). These recombinant cell lines have been particularly useful in defining different homodimeric and heterodimeric combinations of the type 1 receptor kinases that can bind to various ligands within the EGF family of peptides (23). Since CR-1 is structurally related in part to this family of peptides, the 47-mer CR-1 refolded peptide and an Sf9-derived recombinant human GST-CR-1 fusion protein[3] were tested for their ability to directly bind and stimulate receptor tyrosine phosphorylation in single recombinant Ba/F3 derivatives that are expressing different *erb* B family members (Fig. 2). p47 CR-1 was unable to significantly modify EGFR (Fig. 2*A*), *erb* B-2/*neu*/HER-2 (Fig. 2*B*), *erb* B-3/HER-3 (Fig. 2*D*), or *erb* B-4/HER-4 (Fig. 2*C*) tyrosine phosphorylation in Ba/F3 clones that were expressing these tyrosine kinases. In contrast, BTC was able to stimulate EGFR and *erb* B-4 phosphorylation (Fig. 2, *A* and *C*) while HRGβ1 was able to indirectly stimulate *erb* B-2 phosphorylation (Fig. 2*B*) by binding to endogenous *erb* B-3 (22). p47 CR-1 was also ineffective in modulating the tyrosine phosphorylation of these different type 1 receptor tyrosine kinases in Ba/F3 cells that were expressing different pairs of *erb* B receptors demonstrating that CR-1 does not directly bind to heterodimers within this family of receptor tyrosine kinases (data not shown). Similar negative results on tyrosine receptor phosphorylation in either single or double *erb* B expressing recombinant Ba/F3 clones were obtained after treatment of

[3] M. Seno, manuscript in preparation.

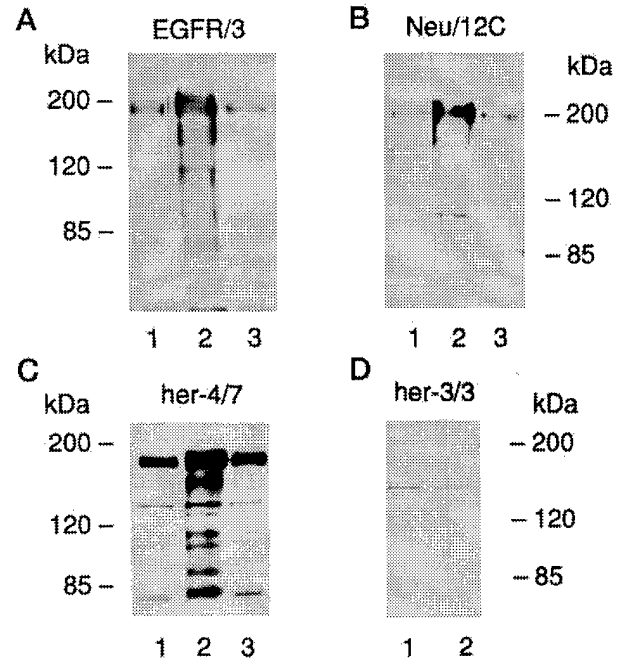

FIG. 2. **Modulation of *erb* B receptor tyrosine phosphorylation by CR-1 in recombinant Ba/F3 clonal derivatives.** Ba/F3 clones that were expressing individual *erb* B family members including the EGFR (*A*), *erb* B-2(*B*), *erb* B-4 (*C*), or *erb* B-3 (*D*) were treated without (*lane 1* in *A–D*) or with 100 ng/ml p47 CR-1 (*lane 3* in *A–C* or *lane 2* in *D*), BTC (*lane 2* in *A* and *C*), or HRGβ1 (*lane 2* in *B*) for 10 min. Cell lysates were then immunoprecipitated with monospecific anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibodies.

these cells with the full-length Sf9 recombinant GST-human CR-1 fusion protein.

CR-1 Stimulates Tyrosine Phosphorylation of Shc and Activates MAPK in HC-11 Mammary Epithelial Cells and in Human Breast Cancer Cells—Although CR-1 does not directly activate any known type 1 receptor *erb* B tyrosine kinase, its receptor may still consist of a membrane-associated tyrosine kinase or alternatively the receptor may associate with a another cytoplasmic or membrane bound tyrosine kinase to generate a signal. To determine if the p47 CR-1 peptide could modulate the tyrosine phosphorylation of other proteins in HC-11 cells, serum-starved cells were treated with p47 CR-1 for 5 min and cell lysates were immunoprecipitated with 4G10 mouse monoclonal anti-phosphotyrosine antibody. Immunoprecipitates were then electrophoresed and probed by Western blot analysis with the PY-20 mouse monoclonal anti-phosphotyrosine antibody (Fig. 3*A*). An increase in tyrosine phosphorylation of a 185-kDa protein was observed. A similar CR-1-induced increase in the tyrosine phosphorylation of a 185-kDa protein was also observed in other cells that were capable of binding ^{125}I-p47 CR-1 such as MDA-MB-453, SKBr3, and T47-D human breast cancer cells after immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies (data not shown). The identity of the p185 phosphoprotein has not yet been identified.

Shc is an SH-2 containing adaptor protein that exists as three distinct isoforms of 66, 52, and 46 kDa and that becomes tyrosine-phosphorylated after ligand activation of several different type 1 receptor tyrosine kinases (27–29). Cell lysates from p47 CR-1-treated serum-starved HC-11 cells exhibited a time-dependent increase in the tyrosine phosphorylation of the p66 Shc and p46 Shc isoforms, which could be detected following immunoprecipitation with an anti-Shc antibody and subsequent screening in Western blot analysis with anti-phosphotyrosine antibodies (Fig. 3, *B* and*C*). In HC-11 cells which had been sufficiently serum-starved, the increase in phosphorylation of p46 Shc (Fig. 3*B*) and p66 Shc (Fig. 3*C*) in response to p47 CR-1 was transient with an 8-fold increase in p46 Shc phosphorylation being observed after 5–7 min, which decreased to control levels after 10 min of treatment (Figs. 3*B* and 5*B*). No significant changes in the phosphorylation of the p52 isoform of Shc were detected in HC-11 cells. A less dramatic but measurable increase in the tyrosine phosphorylation of either p52 and/or p46 Shc could also be detected in either MDA-MB-453 or SKBr-3 human breast cancer cells after treatment with either the p47 CR-1 peptide (Fig. 3*C*) or the GST-CR-1 recombinant fusion protein (data not shown). Proteins of 185 and 120 kDa that were also tyrosine-phosphorylated in a transient manner were detected in the Shc immunoprecipates after probing the Western blots with a mixture of two different anti-phosphotyrosine monoclonal antibodies (Fig. 3*C*). These proteins were found to co-immunoprecipitate with Shc after stimulation of HC-11, MDA-MB-453, or SKBr-3 cells with p47 CR-1. The identity of these two proteins has not been determined.

Shc is an adaptor protein that can bind to specific consensus sequences which contain phosphotyrosine residues in the COOH terminus of several different tyrosine kinase receptors through either an SH2 domain or a phosphotyrosine-binding domain (29). In turn, binding of phosphorylated Shc to the SH2 domain of Grb2 through the tyrosine-phosphorylated collagen homology domain of Shc can link a number of different growth factor receptor tyrosine kinases to the *ras/raf*/MEK/MAPK signaling pathway since Grb2 is intrinsically complexed with the *ras* guanine nucleotide exchanger, SOS (27, 29). To ascertain if p47 CR-1 could facilitate the association of the Grb2-mSOS complex with phosphorylated Shc in mammary epithelial cells, HC-11 cell lysates were immunoprecipated with the Shc antibody and immunoblotted with either an anti-Grb2 (Fig. 4*A*) or an anti-SOS (Fig. 4*B*) antibody. In both cases, p47 CR-1 induced a time-dependent increase in the association of Grb2 and mSOS with phosphorylated Shc. Activation of $p21^{ras}$ by SOS can ultimately lead to the downstream activation of mitogen-activated, *erk*-activating kinase (MEK) through *raf* and the subsequent stimulation of MAPK activity (16, 30). To ascertain if p47 CR-1 could stimulate MAPK activity, serum-starved HC-11 cells were treated for different times with the peptide, and cell lysates were electrophoresed and probed with an anti-MAPK antibody that detects both MAPK isoforms, $p44^{erk\text{-}1}$ and $p42^{erk\text{-}2}$ (Fig. 5*A*). Alternatively, cell lysates were immunoprecipitated with an anti-MAPK antibody and utilized in an *in vitro* kinase assay with MBP as a substrate to quantify total MAPK activity (Fig. 5*B*). The p47 CR-1 peptide produced a rapid tyrosine phosphorylation of $p42^{erk\text{-}2}$ within 3–5 min that could be detected by the presence of a slower migrating band that represents the phosphorylated form of $p42^{erk\text{-}2}$ (Fig. 5*A*). Phosphorylation of this species of $p42^{erk\text{-}2}$ peaked at 7 min and subsequently declined. A nearly 4-fold increase in phosphorylation of the MAPK substrate, MBP, was also observed and found to have identical kinetics in response to p47 CR-1 treatment and was delayed relative to the increase in p46 Shc phosphorylation (Fig. 5*B*).

DISCUSSION

EGF, TGFα, AR, and HRGβ1 stimulate the growth and regulate the differentiation of normal and malignant mouse and human mammary epithelial cells *in vitro* (7, 11, 31–34). However, the role of other EGF-related peptides in regulating the growth and differentiation of mammary epithelial cells has not been fully explored. This may be particularly important since only a subset of peptides within the EGF family bind exclu-

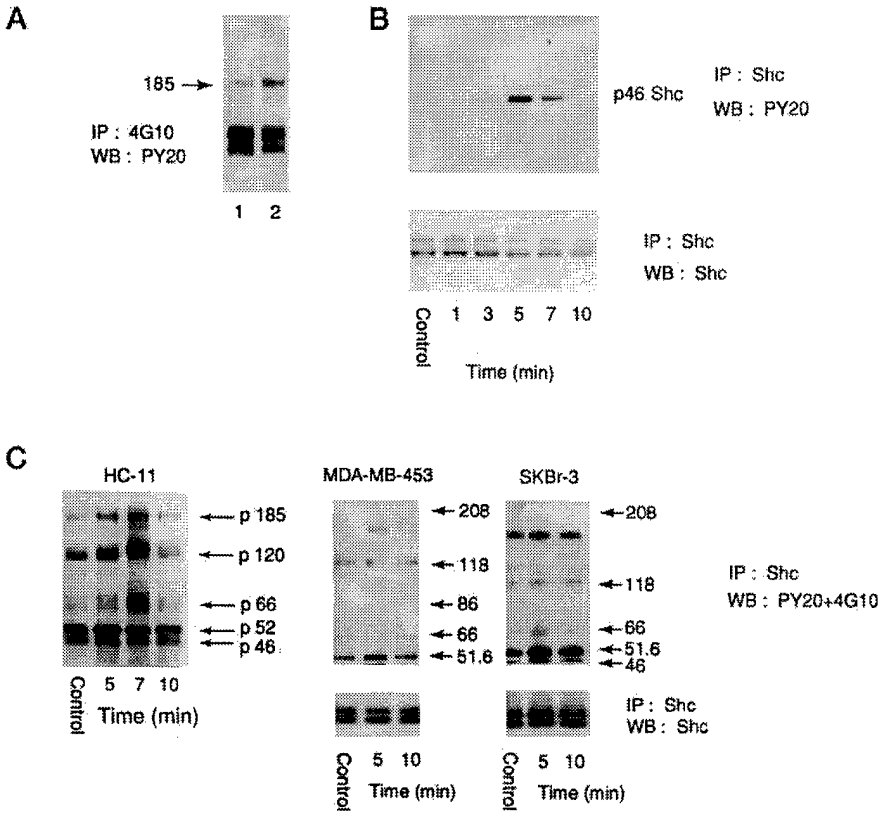

FIG. 3. **Tyrosine phosphorylation of Shc after CR-1 treatment.** Serum-starved HC-11 (*A-C*) or MDA-MB-453 or SKBr-3 human breast cancer cells (*C*) were treated without or with 100 ng/ml p47 CR-1 for 5 min. *A, lane 2*, or various times indicated (*B* and *C*). The cell lysates were immunoprecipitated with anti-phosphotyrosine (4G10, Upstate Biotechnologies) antibody (*A*) or a polyclonal anti-Shc antibody (*B* and *C*). The immunoprecipitates (*IP*) were resolved on a 8–16% SDS-PAGE gel and immunoblotted with a PY20 antibody (*A* and *B, upper panel*) or a mixture of PY20 and 4G10 antibodies (*C*). The *lower panels* in *B* and *C* represent the same blots stripped and reprobed with monoclonal anti-Shc antibody to demonstrate that equal amounts of Shc are present in all lanes.

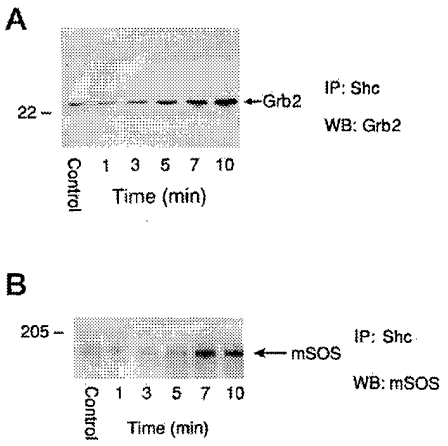

FIG. 4. **Enhanced Grb2-mSOS association with Shc upon CR-1 stimulation in HC-11 cells.** HC-11 cells were treated without or with p47 CR-1 (100 ng/ml) for various times. The cell lysates were immunoprecipated (*IP*) with polyclonal anti-Shc antibody and analyzed by Western blotting using an anti-Grb2 (*A*) or anti-mSOS (*B*) antibody.

sively to the EGFR. Additional proteins in this family, such as the neuregulin subfamily which include the HRGs, bind to other members of the type 1 receptor tyrosine kinase family of receptors such as c-*erb* B-3 or c-*erb* B-4, that can then heterodimerize and activate c-*erb* B-2 following transphosphorylation (3–15, 31, 34–36). Finally, BTC can equally activate either the EGFR or c-*erb* B-4 (23). Since ligand-dependent activation of the EGF receptor can also lead to heterodimerization with c-*erb* B-2, c-*erb* B-3, or c-*erb* B-4, this suggests that different combinatorial pairs of heterodimers within the type 1 receptor tyrosine kinase family may contribute in a hierarchical fashion through signial diversification to the array of responses that are produced to various EGF-like ligands in a cell-specific manner (14, 15, 35, 36).

The present study is the first to demonstrate that a refolded peptide which corresponds in sequence to the EGF-like motif of the human CR-1 protein can bind to a unique receptor that can modify the tyrosine phosphorylation of different proteins which are components in the *ras/raf*/MEK/MAPK pathway. CR-1 is a newly discovered member of the EGF family of peptides that is structurally unique within this family as CR-1 lacks an A loop and possesses a truncated B loop (2, 3, 7). Since conserved amino acid residues in the A loop in conjunction with residues in the C loop are necessary for peptide binding to the EGFR receptor (37) and since the 47-mer refolded CR-1 related peptide does not directly compete with EGF for binding to the EGFR (3), this demonstrates that CR-1 cannot bind to this receptor. Nevertheless, CR-1 is able to stimulate the proliferation and differentiation of HC-11 cells[2] and the growth of several different nontransformed human mammary epithelial and breast cancer cell lines suggesting that a receptor exists for this peptide (3). In this context, the synthetic CR-1 peptide interacts with a high-affinity binding site on HC-11 mouse mammary epithelial cells and on several different human breast cancer cell lines that exhibit specificity for the CR-1 47-mer peptide since other EGF-related peptides that bind either to the EGFR or c-*erb* B-3 and c-*erb* B-4 fail to compete for binding with the labeled p47 CR-1 peptide. This suggests that the CR-1-binding site is unique. Conversely, the CR-1 peptide or recombinant GST-CR-1 fusion protein does not directly activate the tyrosine kinase of either the EGFR or other members of the type 1 receptor tyrosine kinase family either alone or in various heterodimeric combinations in mouse Ba/F3 cells (22, 23). Nevertheless, the ability of 47-mer CR-1 peptide to induce the tyrosine phosphorylation of 185- and 120-kDa proteins in HC-11 mouse mammary epithelial cells and MDA-MB-453 or SKBr-3 human breast cancer cells suggests that CR-1 binds to a potential receptor that has either an intrinsic tyrosine kinase

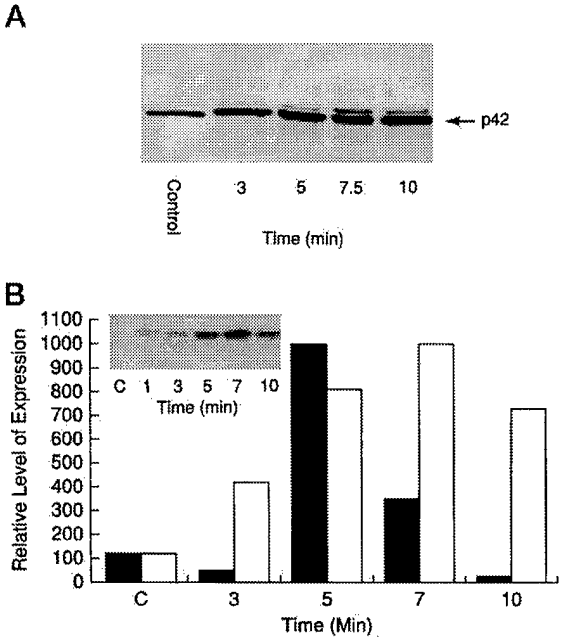

FIG. 5. **Effect of CR-1 on MAPK activation in HC-11 cells.** HC-11 cells were treated for various times with p47 CR-1 (100 ng/ml). In *A* crude cell lysates were run on 10% SDS-PAGE gels and probed with an anti-MAPK antibody (Santa Cruz) to show the hyperphosphorylated, slower migrating band of $p42^{erk\text{-}2}$. In *B*, *insert*, cell lysates were immunoprecipitated with anti-erk-2 antibody (Upstate Biotechnology) and assayed for MAPK activity by an *in vitro* kinase assay using MBP as a substrate followed by electrophoresis and autoradiography. Blots *B* in *3* and *5* were scanned densitometrically using NIH Image program 1.58 and the phosphorylation of Shc (■) and MAPK (□) relative to control are represented graphically.

activity or that associates with a tyrosine kinase. In this context, it is known that various members of the type 1 receptor tyrosine kinase family can heterodimerize following ligand binding or associate with $p60^{c\text{-}src}$. Heterodimerization might facilitate the activation of different signaling proteins thereby contributing to signal amplification and diversification in response to different type 1 receptor ligands (14, 15, 36). The present experiments do not formally exclude the possibility that the CR-1 receptor can also heterodimerize with one of these type 1 receptor tyrosine kinases or with other soluble *src*-related tyrosine kinases and that these interactions may be essential for propagation of an intracellular signal.

The present data also demonstrates that p47 CR-1 treatment of HC-11, MDA-MB-453, or SKBr-3 cells can lead to a rapid increase in tyrosine phosphorylation of the p66, p52, and p46 isoforms of Shc which can then associate with the Grb2-mSOS signaling complex. Since this is one possible mechanism by which other growth factor receptor tyrosine kinases can couple to the MEK/MAPK pathway through *ras* and *raf* (27–30, 38), these results may be functionally significant with respect to defining components in the intracellular signal transduction pathway that are activated by the CR-1 receptor. Moreover, the results demonstrate that CR-1 can activate $p42^{erk\text{-}2}$ MAPK by rapidly inducing the tyrosine phosphorylation of this MAPK isoform. Since activation of MAPK by various growth factors appears in certain cells to be obligatory for cell proliferation and/or for differentiation (16), then this same situation may also be applicable to CR-1 in its ability to stimulate growth and modulate the expression of β-casein and whey acidic protein in HC-11 cells and in primary mouse mammary epithelial cells in response to lactogenic hormones such as prolactin.[2] Identification and characterization of the CR-1 receptor following chemical cross-linking of its ligand should clarify some of these issues. In this respect, attempts to chemically cross-link the ^{125}I-p47 CR-1 peptide have been unsuccessful. However, we have recently expressed a full-length, refolded, biologically active human CR-1 protein in *Escherichia coli* and in Sf9 insect cells with a baculovirus expression vector.[3] Cross-linking of these proteins to appropriate target cells such as HC-11 should now be feasible.

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Ligands for ErbB-family receptors encoded by a neuregulin-like gene

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Neuregulins (also called ARIA[1], GGF[2], heregulin[3] or NDF[4]) are a group of polypeptide factors that arise from alternative RNA splicing of a single gene. Through their interaction with the ErbB family of receptors (ErbB2, ErbB3 and ErbB4), neuregulins help to regulate cell growth and differentiation in many tissues[5–7]. Here we report the cloning of a second neuregulin-like gene, neuregulin-2. The encoded product of the neuregulin-2 gene has a motif structure similar to that of neuregulins and an alternative splicing site in the epidermal growth factor(EGF)-like domain gives rise to two isoforms (α and β). Northern blot and *in situ* hybridization analysis of adult rat tissues indicate that expression of neuregulin-2 is highest in the cerebellum, and the expression pattern is different from that of neuregulins. Recombinant neuregulin-2β induces the tyrosine-phosphorylation of ErbB2, ErbB3 and ErbB4 in cell lines expressing all of these ErbB-family receptors. However, in cell lines with defined combinations of ErbBs, neuregulin-2β only activates those with ErbB3 and/or ErbB4, suggesting that signalling by neuregulin-2 is mediated by ErbB3 and/or ErbB4 receptors.

ErbB2, ErbB3 and ErbB4[8–10] are members of a subfamily of receptor tyrosine kinases that also includes the EGF receptor (EGFR). Signalling through ErbB family receptors is important for regulating cell proliferation and differentiation in many tissues[5–7], and deregulation of these signalling pathways is implicated in a variety of cancers[11]. Although it has been demonstrated that neuregulins can activate ErbB2/3/4 receptors through direct or indirect interaction[12,13], additional ligands for ErbB-family receptors may exist[14–16]. We used a polymerase chain reaction (PCR) based strategy to search for neuregulin-related sequences in an adult rat cerebellum complementary DNA library and have identified a new neuregulin-like gene[17], neuregulin-2.

Figure 1 shows the deduced amino-acid sequence of neuregulin-2β, derived from a composite of two overlapping cDNA clones. This composite contains an open reading frame (ORF) encoding a 754-amino-acid protein. Sequence analysis revealed four structural motifs: a putative signal sequence, a C2-type immunoglobulin-like (Ig-like) domain[18], an EGF-like domain (residues 252–297) with its six characteristic cysteines[19], and a putative transmembrane domain (which separates the whole sequence into a 315-residue extracellular domain and a 414-residue cytoplasmic domain). Another neuregulin-2 cDNA clone, with an extra 77-base-pair (bp) exon inserted between the fourth and fifth cysteine residues of the EGF-like domain, encodes an alternatively spliced variant of neuregulin-2 with a different EGF-like domain (see Supplementary Information). This neuregulin-2 isoform also lacks a transmembrane domain, because the insertion of the extra exon causes a frameshift in the downstream sequence and the termination of the ORF 33 amino acids downstream of the EGF-like domain. Neuregulin-2 molecules having two variant EGF-like domains are termed neuregulin-2α and neuregulin-2β, respectively. The neuregulin gene also has a similar alternative splicing site that gives rise to the α- and β-subtypes of neuregulins[2,3,20], although neuregulin-2α and neuregulin-2β are about equally distant from neuregulin-α or from neuregulin-β. Moreover, there is another alternative splicing site in the cytoplasmic domain of neuregulin-2 in other neuregulin-2 cDNA clones (data not shown), corresponding to the a/b/c-tail splicing site in the neuregulin gene[20]. Therefore, neuregulin-2 and neuregulin not only have similar sequences, they also have similar gene structures.

A protein-database search revealed that the neuregulin-2 proteins are most similar to neuregulins (and to heregulin-β1 among the isoforms of the neuregulins). Overall neuregulin-2β shares 45% identity with heregulin-β1 (ref. 3) and 40% with GGFII (ref. 2). Apart from the N terminus of neuregulin-2β, the similarity between neuregulin-2β and heregulin-β1 extends through their entire sequence (Fig. 2). On the other hand, the N terminus of neuregulin-2β has significant identity to that of GGFII (43%) (Fig. 2). The

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1   MRRDPAPGFSMLLFGVSLACYSPSLKSVQDQAYKAPVVVEGKVQGLAPAGGSSSNSTREP   60
61  PASGRVALVKVLDKWPLRSGGLQREQVISVGSCAPLERNQRYIFFLEPTEQPLVFKTAFA   120
121 PVDPNGKNIKKEVGKILCTDCATRPKLKKMKSQTGEVGEKQSLKCEAAAGNPQPSYRWFK   180
181 DGKELNRSRDIRIKYGNGRKNSRLQFNKVKVEDAGEYVCEAENILGKDTVRGRLHVNSVS   240
241 TTLSSWSGHAFKCNETAKSYCVNGGVCYYIEGINQLSCKCPVGYTGDRCQQFAMVNFSKH   300
301 LGFELKEAEELYQKRVLTITGICVALLVVGIVCVVAYCKTKKQRRQMHHHLRQNMCPAHQ   360
361 NRSLANGPSHPRLDPEEIQMADYISKNVPATDHVIRREAETTFSGSHSCSPSHHCSTATP   420
421 TSSHRHESHTWSLERSESLTSDSQSGIMLSSVGTSKCNSPACVEARARRAAAYSQEERRR   480
481 AAMPPYHDSIDSLRDSPHSERYVSALTTPARLSPVDFHYSLATQVPTFEITSPNSAHAVS   540
541 LPPAAPISYRLAEQQPLLRHPAPPGPGPGPGADMQRSYDSYYYPAAGPGPRRGACALGGS   600
601 LGSLPASPFHIPEDDEYETTQECAPPPPPRPRTRGASRRTSAGPRRWRRSRLNGLAAQRA   660
661 RAARDSLSLSSGSGCGSASASDDDADDADGALAAESTPFLGLRAAHDALRSDSPPLCPAA   720
721 DSRTYYSLDSHSTRASSRHSRGPPTRAKQDSGPL  754
```

Figure 1 Deduced amino-acid sequence of rat neuregulin-2β. Arrowed underline marks the putative signal sequence. The immunoglobulin-like domain is outlined by a dashed box. Solid frame surrounds the EGF-like domain; the six cysteines characteristic of this domain are indicated by asterisks. Potential *N*-glycosylation sites are marked with arrowheads. The putative transmembrane region is underlined. An arrow points to the potential proteolytic site.

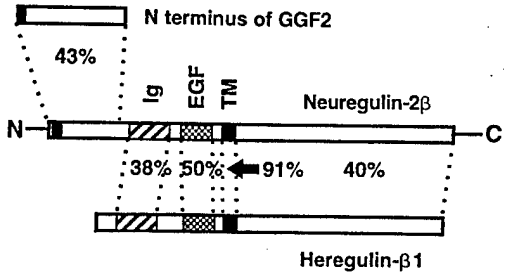

Figure 2 The motif structure of the neuregulin-2β and its similarity to selected members of neuregulins. The percentage similarity is calculated from the amino-acid sequence alignment of neuregulin-2β, heregulin-β1 (human)[3] and the N terminus of GGFII (human)[2]. Black boxes, potential signal sequences; Ig, immunoglobulin-like domains; EGF, EGF-like domains; TM, transmembrane domains.

most similar region between neuregulin-2β and heregulin-β1 is the transmembrane domain (91% identity) and adjacent sequence. Highly conserved regions also exist in the cytoplasmic tails of neuregulin-2β and heregulin-β1, indicating that the cytoplasmic domains may be important biologically. Relatively high conservation between neuregulin cytoplasmic tails from distant vertebrate species has been noted before[1]. As the EGF-like domain of neuregulins is sufficient for receptor binding and for stimulating cellular responses[3], we compared the EGF-like domain of neuregulin-2 molecules with other EGF-like motifs. Among the known EGF-like motifs, the EGF-like domain of neuregulin-2 is most similar to that of the neuregulins (48% identity between terminal cysteines in the case of heregulin-β1). Second to neuregulins is the rat epidermal growth factor, with 43% identity between terminal cysteines.

To determine the size and tissue distribution of neuregulin-2 mRNAs, northern blot hybridization with poly(A)$^+$ RNA was carried out using a probe spanning the EGF-like domain plus the immunoglobulin-like domain (Fig. 3A). Among the adult rat tissues examined, neuregulin-2 transcripts were most abundant in neural tissues (brain and spinal cord) and lung. A separate experiment with total RNA samples shows that the cerebellum has the highest concentration of neuregulin-2 transcripts compared to other parts of the brain and other adult tissues (data not shown). Three bands were seen in brain samples (Fig. 3A): a prominent band of 3 kilobases (kb), and two additional bands of 3.8 and 6 kb. Only the 3- and 3.8-kb transcripts were detected in spinal cord and lung samples. This pattern of three principal transcripts has also been found for the neuregulin gene, but at the sensitivity of the northern blot, the tissue distribution of neuregulin-2 transcripts in adult rat seems to be more restricted than that of neuregulins[3,4].

We also characterized neuregulin-2 expression by *in situ* hybridization with several probes. In adult rat brain sections, the highest expression was detected in the cerebellum (in the Purkinje cell layer and the granule cell layer) and in the dentate gyrus of the hippocampus (Fig. 3B). Labelled cells were also found in the olfactory bulb (data not shown). This expression pattern seems to be distinct from that of neuregulins, because no hybridization signal for neuregulins is observed in Purkinje cells and very little in the granule cell layer[21]. We investigated the expression pattern of

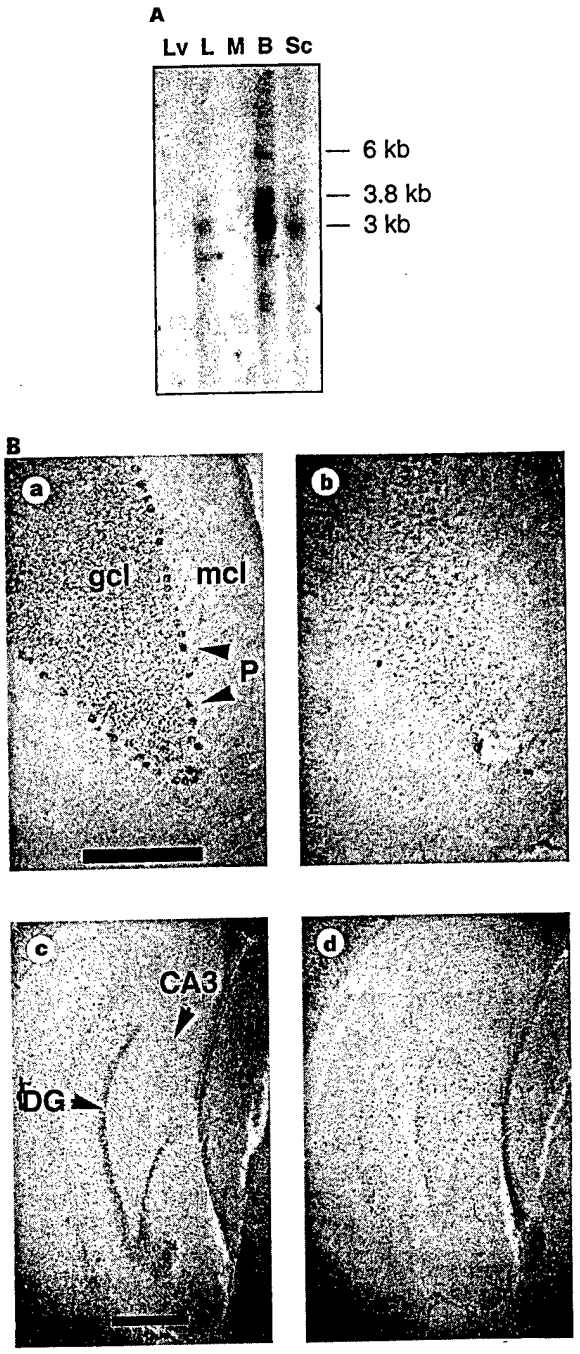

Figure 3 Expression of neuregulin-2 transcripts in adult rat tissues. **A**, Northern blot analysis using poly(A)$^+$ RNA samples; approximately 2 μg poly(A)$^+$ RNA was loaded in each lane. The three bands detected (3, 3.8, 6 kb) are indicated. Lv, liver; L, lung; M, skeletal muscle; B, brain; Sc, spinal cord; **B**, *In situ* hybridization of adult rat brain parasagittal sections with a digoxingenin-labelled cRNA probe spanning the EGF-like and Ig domains. **a**, Neuregulin-2 transcripts were detected in Purkinje cells (P) and in the granule cell layer (gcl) in the cerebellum, but not in the molecular cell layer (mcl); scale bar, 0.4 mm. **b**, Adjacent section hybridized with a sense control probe. **c**, In the hippocampus, neuregulin-2 transcripts were only detected in the dentate gyrus (DG) but not in the CA1–CA3 area; scale bar, 0.8 mm. **d**, Adjacent section hybridized with the sense control probe.

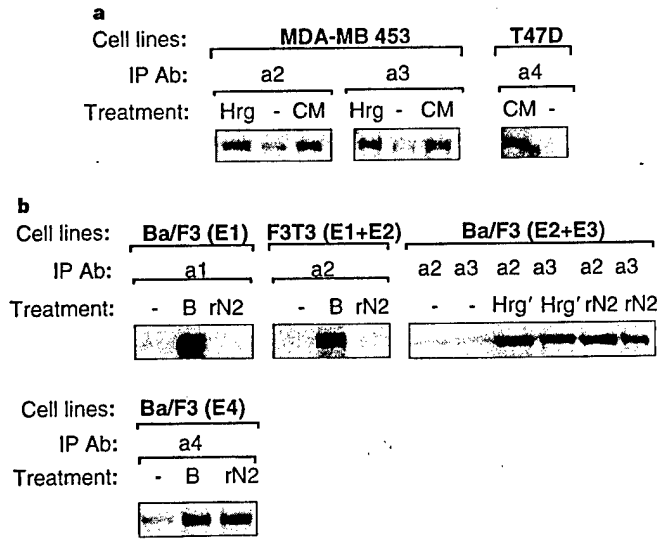

Figure 4 Recombinant neuregulin-2β protein induces tyrosine-phosphorylation of ErbB-family receptors through ErbB3 and ErbB4. **a**, Neuregulin-2β induces tyrosine phosphorylation of ErbB2, ErbB3 and ErbB4 in MDA-MB 453 and T47D cell lines. **b**, Neuregulin-2β signalling through ErbB3 and ErbB4 receptors. rNRG-2β was tested on cell lines transfected with defined ErbB-family receptors. Only cells with ErbB3 and/or ErbB4 receptors were activated. E1, EGF receptor; E2, ErbB2; E3, ErbB3; E4, ErbB4. Immunoprecipitating antibodies (IP Ab): a1, anti-EGF receptor; a2, anti-ErbB2; a3, anti-ErbB3; a4, anti-ErbB4. B, betacellulin; Hrg', heregulin-β1 EGF-like domain; rN2, rNRG-2β (EGF-like domain of neuregulin-2β); CM, neuregulin-2β-conditioned medium; −, negative control.

neuregulin-2 during embryogenesis by whole-mount *in situ* hybridization with the same probes. We did not detect any signal in E9.5–E10 mouse embryos (data not shown), indicating that none or very little neuregulin-2 is expressed in embryos at those developmental stages. Therefore neuroregulin-2 is unlikely to be the 'missing ligand' for ErbB4 receptors[15].

The structural similarity between neuregulin-2 and neuregulins suggests that the neuregulin-2 proteins may also function as ligands for ErbB family receptors. To test this, we expressed a large portion of neuregulin-2β (including all of the extracellular domain and part of cytoplasmic domain) in CHO cells. As the sequences around the putative proteolysis sites are highly conserved between neuregulin-2 and neuregulins, a soluble form of neuregulin-2β protein should be released from its precursors to the culture medium, as in the case of neuregulins[1–4]. We treated cells expressing ErbB family receptors (MDA-MB453 and T47D breast cancer cell lines)[22,23] with conditioned medium from stably transfected CHO cells. As shown in Fig. 4a, ErbB2, ErbB3 and ErbB4 receptors were activated by neuregulin-2β-conditioned medium. But as ErbB family receptors can form ligand-induced heterodimers, the activation of ErbB2/3/4 receptors could be due to direct or indirect interaction with neuregulin-2β. We also expressed the EGF-like domain (amino acids 240–305; Fig. 1) of neuregulin-2β in bacteria and produced a refolded neuregulin-2β protein fragment (rNRG-2β) from inclusion bodies. rNRG-2β can activate ErbB-family receptors in our breast-tumour cell lines (data not shown), suggesting that like neuregulins, the EGF-like domain is the functional domain for activating ErbB-family receptors.

To determine which of the ErbB family receptors is involved in neuregulin-2β signalling, we tested rNRG-2β on cell lines expressing defined combinations of ErbB receptors. We did not detect rNRG-2β activation of EGF receptors in the Ba/F3 (EGFR) cell line or of ErbB2 receptor in the Fischer rat 3T3 cell line (Fig. 4b), whereas our positive control, betacellulin[24], stimulated these receptors. On the other hand, rNRG-2β stimulated ErbB4 receptor in the Ba/F3 (ErbB4) cell line, as well as ErbB2 and ErbB3 receptors in the Ba/F3 (ErbB2 + ErbB3) cell line. These results indicate that neuregulin-2β signalling results from direct interaction with ErbB3 and/or ErbB4 receptors.

We have shown that the neuregulin-2 gene, which has structural similarity to the neuregulin gene, encodes new ligands for the ErbB3 and ErbB4 receptors. The distinct expression pattern of neuregulin-2 suggests that these proteins have specific biological functions. It will be necessary to compare neuregulin-2 with neuregulins and other ligands for ErbB-family receptors, including the temporal and spatial regulation of their expression, in order to understand the function of this multiligand/multireceptor signalling network.

Methods

Cloning of neuregulin-2 cDNAs. Two pools of degenerate oligonucleotides were synthesized based on two conserved regions of the neuregulin sequences, one in the immunoglobulin-like domain and the other in the EGF-like domain. Phages from an adult rat cerebellum cDNA library (gift from D. Zhao) were used as templates for PCR. Two steps were used to reduce neuregulin sequences and select neuregulin-related sequences. First, PCR products were digested with *BclI* and separated by agarose gel electrophoresis, because there is a *BclI* site in rat neuregulin cDNA[4]. DNA fragments of expected sizes were isolated from the agarose gel and reamplified with the same primers. Final PCR products were subcloned into pBlueScriptII vector (Stratagene). Second, individual clones were hybridized with a neuregulin probe under low-stringency conditions and positive clones were sequenced. We identified one clone, n9, which has significant homology to neuregulins. ^{32}P-labelled probes from the n9 insert were used to screen the cDNA library (~500,000 clones) and several positive clones were identified. The insert of each clone was sequenced in both directions and analysed.

Northern blot and *in situ* hybridization. Poly(A)$^+$ RNA was purified from tissues by using a FastTrack kit (Invitrogen). RNA samples were separated on agarose gels and transferred to nylon filters by standard procedures. Filters were hybridized with ^{32}P-labelled probes under high-stringency conditions. A probe was generated by random priming of a fragment of neuregulin-2 cDNAs spanning the EGF-like plus the Ig-like domains. The highly conserved transmembrane domain and adjacent sequence were excluded. The probe would hybridize to both neuregulin-2α and neuregulin-2β transcripts. *In situ* hybridization was done essentially as described[25]. We used a digoxigenin-labelled cRNA probe spanning the EGF-like plus the Ig-like domains. Several other probes derived from different parts of neuregulin-2 cDNAs (that is, the EGF-like domain only, the Ig domain only) also gave essentially the same hybridization pattern.

Expression of recombinant neuregulin-2 proteins. The insert of a partial neuregulin-2β cDNA clone was subcloned into the pRc/CMV expression vector (Invitrogen) and stably transfected into CHO cells. Serum-free conditioned medium was collected. Negative control media were conditioned medium from CHO cells or from CHO cells transfected with an unrelated gene. For expression of rNRG-2β in *E. coli*, the EGF-like domain of neuregulin-2β (residues 240–305) was sublconed into pQE32 expression vector with an N terminus 6 × histidine tag (Qiagen). Protocols for solubilization and refolding of proteins from inclusion bodies were essentially as described[24], except that refolded proteins were not purified further. The final rNRG-2β protein concentration is ~500 μg ml^{-1}.

Tyrosine-phosphorylation assay. MDA-MB 453 and T47D cells were starved in serum-free medium for 2–6 h before addition of neuregulin-2β conditioned medium, negative control medium, or heregulin-β1 (extracellular portion, 20 ng ml^{-1}; from S. J. Burden). After 5–10 min at room temperature, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 SDS, 1 mM sodium orthovanadate, 50 μg ml^{-1} aprotinin, 0.5 mM PMSF), immunoprecipitated with rabbit antibodies (Santa Cruz Biotechnology) specific for ErbB2 (C18), ErbB3 (C17) or ErbB4 (C18). Immunoprecipitated proteins were collected on protein A–Sepharose beads, analysed by western blotting with an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). Antibody binding was detected by enhanced chemiluminescence (Amersham Life Science). The recombinant Ba/F3 cell lines expressing ErbB family receptors have been described, as have protocols for stimulating and analysing ErbB-family receptor tyrosine-phosphorylation[24,26,27]. EGFR and ErbB2 expression in Fischer rat 3T3 (F3T3) was described[28]. Human recombinant betacellulin (R&D Systems) was used at 200 ng ml^{-1}. Chemically synthesized heregulin-β1 65-mer[29] was used at 94 ng ml^{-1}. Although we can detect the activity of rNRG-2β at a dilution of 1:50,000, it was routinely used at a dilution of 1:100 to ensure saturated receptor phosphorylation.

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Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases

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The neuregulins (NRGs) are a family of multipotent epidermal-growth-factor-like (EGF-like) factors that arise from splice variants of a single gene. They influence the growth, differentiation, survival and fate of several cell types. We have now discovered a set of new neuregulin-like growth factors, which we call neuregulin-2 (NRG-2): these are encoded by their own gene and exhibit a distinct expression pattern in adult brain and developing heart. Like NRG-1, the EGF-like domain of the new ligands binds to both the ErbB3- and ErbB4-receptor tyrosine kinases. However, NRG-2 stimulates different ErbB-receptor tyrosine-phosphorylation profiles from NRG-1. Our results indicate that NRG-1 and NRG-2 mediate distinct biological processes by acting at different sites in tissues and eliciting different biochemical responses in cells.

The neuregulins (NRG-1) are a family of polypeptide growth factors that are thought to be critical for the developing heart and nervous system, and in the generation and progression of tumours. In the peripheral nervous system, neuronally derived NRG-1 acts at the neuromuscular junction to promote the end-stage differentiation of muscle cells[1–4], and in developing nerves to promote the proliferation, survival and differentiation of Schwann cells[4–6]. NRG-1 is also essential in the developing heart: neuregulin-deficient mice fail to form ventricular trabeculae and die in mid-gestation[7–10]. NRG-1 may also play a role in oncogenesis as it can indirectly activate the ErbB2-receptor protein-tyrosine kinase (PTK) whose overexpression correlates with a poor prognosis of some cancer patients[11]. The molecular cloning of these bioactivities has revealed that NRG-1, separately identified as glial growth factors (GGFs), acetylcholine-receptor-inducing activity (ARIA), heregulins (HRGs), and neu differentiation factor (NDF), are the alternatively spliced products of a single gene[12–15]. NRG-1 binds to ErbB3 and ErbB4 (refs 16–19), two members of the ErbB subfamily of receptor PTKs, and stimulates the tyrosine phosphorylation of other ErbB receptors through receptor heterodimerization[19–22].

We have identified a new NRG-1-like gene called neuregulin (NRG-2). Three distinct complementary DNAs were obtained by screening an adult mouse brain cDNA library with a NRG-1 probe; a composite amino-acid sequence is presented in Fig. 1a. NRG-2 is distantly related to NRG-1 (~35% identity) but exhibits a similar overall domain structure. It includes a single EGF-related motif with the same spacing between the third and fourth cysteine residues as that for EGF (and related molecules that bind to the EGF receptor) but distinct from NRG-1 (see Supplementary Information). NRG-2 carries a single immunoglobulin (Ig)-like domain that is ~36% identical to similar domains found in a subset of NRG-1 splice variants. The transmembrane segment is highly conserved (Fig. 1a, b) and, by analogy with NRG-1, cleavage of the NRG-2 precursor may yield a soluble NRG-2 isoform. The structure of the NRG-2 cDNAs indicates that alternative splicing similar to those found in NRG-1 (refs 12, 14) also generates multiple NRG-2 EGF-like isoforms, including a β1-like (clone 16A) and an α-like form (clone 5). The splicing alters the carboxy-terminal portion of the EGF-like motif; the NRG-2 and NRG-1 β1-like forms are particularly highly conserved in this region, with 16/28 identical residues.

To identify sites of NRG-2 expression we analysed, by northern blotting, messenger RNA samples from a series of adult rat tissues (Fig. 2a). Neural expression of NRG-2 mRNA was observed primarily in the cerebellum and olfactory bulb; a transcript was also detected in liver. By whole-mount *in situ* hybridization in mouse E9.5 embryos, NRG-2 mRNA was detected in the endothelial lining of the heart, with the highest mRNA levels being found in the atrium and lower levels in the ventricle and outflow tract (Fig. 2b, c). This profile is complementary to that of NRG-1, which is expressed at high levels in the ventricular endothelium and only weakly in the atrial endothelium[7]. It also differed from NRG-1 in the developing hindbrain, where NRG-1 has been identified in rhombomeres 2, 4 and 6 (ref. 7) and NRG-2 was not detected. In the adult rat, expression of NRG-2 and NRG-1 mRNA was compared by *in situ* hybridization. In the cerebellum, NRG-2 mRNA was detected in granule and Purkinje cells (Fig. 2k), whereas NRG-1 was found mainly in Golgi II cells[23,24] (Fig. 2j). Cerebellar granule cells also express ErbB4 mRNA (C.L., unpublished results), suggesting a possible autocrine role for NRG-2. In the hindbrain, the NRG-2 mRNA was weakly detectable in the motor trigeminal nucleus (Fig. 2i), compared to a strong NRG-1 signal (Fig. 2h). In the hippocampus, granule cells of the dentate gyrus express NRG-2 (Fig. 2g), where no NRG-1 was detected (Fig. 2f). The cholinergic cells in the basal forebrain that express NRG-1 mRNA[23,24] (Fig. 2d) are only faintly positive for NRG-2 (Fig. 2e). NRG-2 mRNA was not detected in the hypothalamus[23,24], which produces the pituitary-derived NRG-1 known as GGF (not shown). These profiles indicate that in the adult brain NRG-1 and NRG-2 are expressed in largely non-overlapping neural cell populations, suggesting that the two ligands have distinct functional roles.

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Activation of ErbB4 by the Bifunctional EGF Family Hormone Epiregulin is Regulated by ErbB2

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Summary

Epiregulin (EPR) is a recently-described member of the epidermal growth factor (EGF) family of peptide growth factors. The ever-expanding size of the EGF family has made distinguishing the activities of these hormones paramount. We show here that EPR activates two members of the ErbB family of receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ErbB4. Therefore by these criteria, EPR is qualitatively similar to another EGF family hormone, betacellulin (BTC). Yet, here we also demonstrate quantitative differences between EPR and BTC. EPR stimulates higher levels of EGFR phosphorylation than does BTC, while BTC stimulates higher levels of ErbB4 phosphorylation than does EPR. Moreover, the EPR and BTC dose response curves show that while EGFR is more sensitive to EPR than is ErbB4, ErbB4 is more sensitive to BTC than is EGFR. Finally, ErbB2, which is not activated by EPR when expressed on its own, increases the sensitivity of ErbB4 for activation by EPR Therefore, these results establish that EPR exhibits novel activities and modes of regulation, which may have significant implications for EPR function *in vivo*.

Introduction

The continuing discovery of novel members of the epidermal growth factor (EGF) family of peptide growth factors has led to an increased appreciation of the functional differences among these hormones, as well as a realization of the complex hormone-receptor interactions fostered by these peptides. EGF, transforming growth factor alpha (TGFα), and amphiregulin (AR) all bind exclusively to the EGF receptor (EGFR). Yet, these hormones can also activate in *trans* (transmodulate) the other three ErbB family receptors (Neu/ErbB2/Her2, ErbB3/Her3, ErbB4/Her4) through ligand-induced receptor heterodimerization with the EGFR [1-8]. Other EGF family hormones bind multiple receptors. Neuregulin (NRG) and Neuregulin2 (NRG2) bind ErbB3 and ErbB4 and transmodulate EGFR and ErbB2 [9-16]. Betacellulin (BTC) combines some of the properties of EGF and NRG by activating EGFR and ErbB4 [7].

EPR was initially purified from the conditioned medium of a tumorigenic clone of NIH3T3 fibroblasts. It competes with EGF for binding to A431 cells, which overexpress EGFR, suggesting that EPR is a ligand for EGFR [17]. Since at least one of the EGF family hormones that activates EGFR also activates ErbB4, we wished to evaluate EPR function in a set of cell lines expressing all four ErbB family receptors, both singly and in every pairwise combination.

We demonstrate here that EPR activates not only EGFR, but ErbB4 as well. However, the dose-response curves for BTC and EPR in a cell line expressing both EGFR and ErbB4 are markedly different. While ErbB4 is more responsive to BTC than is EGFR, ErbB4 is less responsive to EPR than is EGFR. Moreover, ErbB2 expression increases saturated ErbB4 phosphorylation in response to EPR and dramatically enhances the sensitivity of ErbB4 for activation by EPR as well. In this respect EPR resembles NRG, which displays a low affinity for ErbB3 that increases in cells where ErbB2 is co-expressed [12].

Experimental Procedures

Cell Lines and Cell Culture

BaF3 is an immortal mouse lymphoblastoid cell line [31]. BaF3-derived cell lines expressing combinations of ErbB family receptors have been described previously [14]. CEM is an immortal human T-lymphoblastoid cell line which does not endogenously express EGF receptor, ErbB2, ErbB3, or ErbB4. CEM-derived cell lines expressing ErbB4 or ErbB2 and ErbB4 have been described previously [10]. Cell culture conditions were as described [10,14].

Growth Factors

Recombinant human EPR was produced in *Bacillus brevis* [Nakazawa, *et al.*, in preparation]. Recombinant NRGβ was the generous gift of Kerry Russell and Jeffrey Bender (Yale University). We are grateful to Jim Moyer, Brad Guarino, and Glenn Andrews (Pfizer Central Research, Groton, CT) for synthetic NRGβ [32]. Recombinant BTC was purchased from R&D Systems (Minneapolis, MN, USA), while recombinant TGFα was purchased from Collaborative Biomedical Products (Bedford, MA).

Stimulation and Analysis of Receptor Phosphorylation

The conditions for stimulation of ErbB family receptor tyrosine phosphorylation have been described previously [7, 14]. The analysis of ErbB family receptor tyrosine phosphorylation by immunoprecipitation and anti-phosphotyrosine immunoblotting has been described previously [7,14]. Immunoprecipitating anti-receptor antibodies were anti-EGFR mouse monoclonal antibody 528 [33], anti-ErbB2 mouse monoclonal antibody TA-1 (OP-39, Calbiochem), anti-ErbB3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology), and anti-

ErbB4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of anti-receptor antibodies has been verified by testing for cross-reactivity (data not shown).

Immunoblot autoradiographs were digitized using a Hewlett-Packard 3p flat-bed scanner set for 600dpi resolution and controlled by Hewlett-Packard Deskscan II for Macintosh software. Images were cropped using Adobe Photoshop and band intensity was quantified using NIH Image software. Net receptor activation was calculated by subtracting the amount of tyrosine phosphorylation observed in samples from mock-stimulated cells.

Results

EPR activates EGFR

We first sought to identify which ErbB family receptors are activated by EPR when the receptors are expressed individually. We previously developed a panel of cell lines based on the mouse BaF3 hematopoietic cell line that expresses the four ErbB family receptors, both singly and in every pairwise combination. Hence, we incubated with EPR the BaF3 cell lines ectopically expressing EGFR, ErbB2, or ErbB4. EPR, like BTC, stimulated EGFR tyrosine phosphorylation, consistent with published results suggesting that EPR binds EGFR [17] (Fig. 1, "EGFR" panel; compare lanes "E" and "B" with "M). However, EPR did not stimulate phosphorylation of ErbB2 or ErbB4 (Fig 1, "ErbB2" and "ErbB4" panels; compare lanes "E" and "M"). In contrast, the positive control NRGβ stimulated ErbB2 tyrosine phosphorylation and both NRGβ and BTC stimulated ErbB4 phosphorylation. The ErbB2 phosphorylation observed in BaF3/ErbB2 cells stimulated with NRG is the result of transmodulation of ErbB2 by the NRG receptor ErbB3, which is endogenously expressed at low levels in BaF3 cells [14]. Neither EPR nor any of the other EGF family ligands tested to date stimulated ErbB3 tyrosine phosphorylation in BaF3 cells expressing only ErbB3 (data not shown) [7,8,14]. However, since ErbB3 lacks tyrosine kinase activity [18], these experiments do not rule out EPR binding to ErbB3.

Since EPR activates EGFR, we next determined whether EPR activates the other three ErbB family receptors *in trans* via EGFR. A panel of BaF3 cell lines ectopically expressing EGFR together with one of the other three ErbB family receptors was stimulated with EPR. EPR activated the EGFR in all three cell lines (Fig. 2a; compare "E" "α1" lanes with the "M" "α1" lanes). Both EPR ("E" lanes) and the positive control TGFα ("T" lanes) strongly activated ErbB2 in the cell line coexpressing EGFR+ErbB2 (Fig. 2a, "EGFR+ErbB2" panel; "α2" lanes). In contrast,

neither EPR not TGFα activated ErbB3 or ErbB4 (Fig. 2a, "EGFR+ErbB3" and "EGFR+ErbB4" panels; "α3" or "α4" lanes). This is consistent with the conclusion that ErbB2 is a preferential target for transmodulation by the EGFR compared to the other ErbB family receptors [7,8,19-21]. However, higher concentrations of EPR than those used for these experiments did stimulate ErbB4 phosphorylation in the EGFR+ErbB4 cell line (see below, Fig. 5a).

Since ErbB3 lacks functional kinase activity, EGF family hormones can activate ErbB3 only in the presence of another ErbB family receptor, particularly ErbB2, which permits the highest levels of ErbB3 phosphorylation by NRG [11,12,14]. Therefore, BaF3 cells expressing both ErbB2 and ErbB3 or both ErbB3 and ErbB4 were stimulated with EPR to determine if ErbB3 is a receptor for EPR. In the ErbB2+ErbB3 cell line, the positive control NRGβ activated both receptors (Fig 2b, "ErbB2+ErbB3" panel, compare "N" lanes with "M" lanes), while in the ErbB3+ErbB4 cell line NRGβ stimulated a marked increase in ErbB3 phosphorylation and modest increase in ErbB4 phosphorylation (Fig 2b, "ErbB3+ErbB4" panel, compare "N" lanes with "M" lanes). In contrast, EPR did not stimulate receptor phosphorylation in either of these cell lines, suggesting that ErbB3 is not a receptor for EPR (Fig. 2b; compare "E" lanes with "M" lanes).

EPR activates ErbB4 in CEM cells

BTC activates both ErbB4 and EGFR when expressed individually [7]. We tested whether EPR behaves like BTC and also activates ErbB4 expressed alone using derivatives of the CEM human T-lymphoblastoid cell line that ectopically expresses ErbB4 alone or both ErbB2 and ErbB4 [10]. EPR activated ErbB4 in CEM cells expressing ErbB4 alone and both receptors in CEM cells expressing ErbB2 and ErbB4 together (Fig. 3; compare "E" lanes to "M" lanes). In experiments done in parallel using identical growth factor concentrations, EPR did not activate ErbB4 in BaF3

cells expressing ErbB4 alone (also see Fig. 1) but stimulated ErbB2 and ErbB4 phosphorylation in BaF3 cells expressing both ErbB2 and ErbB4 (data not shown). It is unclear why EPR failed to activate ErbB4 in the BaF3 cells expressing ErbB4 alone. Nonetheless, because EPR activates EGFR as well as ErbB4, EPR resembles BTC, which also activates these receptors [7].

EPR stimulates EGFR more than ErbB4 and EGFR is more sensitive than ErbB4 to EPR

Since EPR can activate EGFR and ErbB4 when the receptors are expressed individually, we compared EGFR and ErbB4 phosphorylation in BaF3/EGFR and CEM/ErbB4 cells stimulated with increasing concentrations of EPR (Fig 4a). At saturation, EPR stimulated a slightly higher level of EGFR phosphorylation (1.7 fold) than did BTC, while EPR stimulated a much lower level of ErbB4 phosphorylation (0.3 fold) than did BTC (Fig. 4c). We next compared the dose sensitivity of EGFR and ErbB4 to EPR stimulation by plotting receptor phosphorylation relative to the maximal amounts of receptor phosphorylation stimulated by EPR (Fig. 4d) to identify the EPR concentrations required for half-maximal receptor phosphorylation (Table 1). Half-maximal EGFR phosphorylation occurred at an EPR concentration of approximately 380ng/ml (Fig. 4d; Table 1). In contrast, half-maximal ErbB4 phosphorylation required about a 4-fold higher concentration of EPR, with half-maximal activation occurring at an EPR concentration of approximately 1790ng/ml (Fig. 4d; Table 1).

ErbB2 expression increases ErbB4 activation by EPR and sensitivity to EPR

The affinity of NRG for cells expressing ErbB3 is greater when these cells also express ErbB2 [12]. Hence, we evaluated the possibility that ErbB2 modulates ErbB4 activation by EPR by stimulating CEM/ErbB4 and CEM/ErbB2+ErbB4 cells with

increasing concentrations of EPR (Fig. 4a-b). Relative to the BTC positive control, EPR stimulated 2-fold higher levels of ErbB4 phosphorylation in the ErbB2+ErbB4 cell line than in cells expressing ErbB4 alone (Fig. 4c). Therefore, ErbB2, which is not activated by EPR when expressed by itself (Fig. 1), doubles the magnitude of ErbB4 activation by EPR at saturation. We next examined the effects of ErbB2 expression on the sensitivity of ErbB4 to EPR (Fig. 4d). Half-maximal ErbB4 phosphorylation occurred at an EPR concentration of approximately 1790ng/ml in the cell line expressing ErbB4 alone (Fig. 4d; Table 1), but occurred at an EPR concentration of approximately 630ng/ml (Fig. 4d; Table 1) in the ErbB2+ErbB4 cell line. This shift in the EPR dose-response curve in the ErbB2+ErbB4 cell line compared to the ErbB4 cell line suggests that ErbB2 expression increases the affinity of ErbB4 for EPR and implies that ErbB2-ErbB4 heterodimers have a higher affinity for EPR than do ErbB4-ErbB4 homodimers.

The EPR and BTC dose response curves are different in cells expressing EGFR and ErbB4

EPR resembles BTC in its ability to activate either EGFR or ErbB4 when expressed individually [7]. Yet, at saturation EPR stimulated almost 2-fold more EGFR phosphorylation than BTC, while BTC activated about 3-fold more ErbB4 phosphorylation than did EPR (Fig. 4a, 4c). This suggested that BTC and EPR are functionally distinct. Hence, we compared EGFR and ErbB4 phosphorylation following stimulation with increasing concentrations of BTC or EPR in a BaF3 cell line that expresses both EGFR and ErbB4 (Fig 5a).

We first compared the magnitude of receptor phosphorylation stimulated by EPR and BTC by plotting receptor phosphorylation relative to the maximal phosphorylation stimulated by BTC (Fig. 5b-c). In agreement with results presented above (Fig. 4a, 4c), EPR stimulated higher saturated levels of EGFR phosphorylation

than BTC, while BTC activated greater ErbB4 phosphorylation than did EPR (Fig. 5a, 5c). However, the magnitude of these differences was much less in the EGFR+ErbB4 cell line compared to the differences in phosphorylation we observed between the cell lines expressing EGFR and ErbB4 individually (Fig. 4a, 4c).

Next, we compared the sensitivities of EGFR and ErbB4 to BTC and EPR by identifying the growth factor concentrations required for half-maximal receptor phosphorylation. Half-maximal EGFR activation occurred at a BTC concentration of approximately 35ng/ml, while half-maximal ErbB4 activation occurred at a BTC concentration of approximately 5ng/ml (Fig. 5b; Table 1). In contrast, half-maximal EGFR activation occurred at an EPR concentration of approximately 320ng/ml, while half-maximal ErbB4 activation occurred at an EPR concentration of approximately 790ng/ml (Fig. 5c; Table 1). This suggests that ErbB4 is 7-fold more sensitive to BTC than is EGFR, while EGFR is more than two-fold more sensitive to EPR than is ErbB4.

Finally, these results illustrate that EGFR expression, like ErbB2 expression, shifts the EPR dose-response curve in cells expressing ErbB4. Half-maximal ErbB4 phosphorylation in a CEM cell line expressing ErbB4 alone occurs at an EPR concentration of 1790ng/ml (Fig. 4d; Table 1). In contrast, half-maximal ErbB4 phosphorylation in BaF3 cells expressing both EGFR and ErbB4 occurs at 790ng/ml (Fig. 5c; Table 1).

EPR activates ErbB family receptor coupling to IL3-independence

While EPR and BTC stimulate qualitatively identical patterns of receptor phosphorylation, these hormones are quantitatively distinct. One possible mechanism is that EPR and BTC stimulate EGFR and ErbB4 tyrosine phosphorylation at different sites. This would account for the higher levels of EGFR activation by EPR compared to BTC and the higher levels of ErbB4 activation by BTC

compared to EPR. Moreover, this would also enable these hormones to couple to distinct receptor effectors and physiologic responses. Therefore, we compared EPR and BTC induction of receptor coupling to physiologic responses. BaF3 cells require interleukin-3 (IL3) for survival and for proliferation. However, activation of either EGFR or ErbB2 permits survival of BaF3 cells in the absence of IL3 [7,14]. EPR, like BTC [7], induces IL3-independent survival in BaF3 cells expressing EGFR, but not in vector control BaF3 cells or cells expressing ErbB2 (Fig. 6). (The IL3-independent response of BaF3 cells expressing ErbB2 to NRG is the result of ErbB2 transmodulation by endogenous ErbB3 in these cells [14].) Both EPR and BTC induced IL3 independence in cells co-expressing ErbB2 and ErbB4, presumably through ErbB2 transmodulation by ErbB4 (Fig. 6). Nonetheless, the response to BTC and NRG is greater than the response to EPR, which may reflect subtle functional differences between BTC and EPR.

Discussion

We previously demonstrated that the EGF family of peptide growth factors can be divided into three distinct functional groups [8] (Figure 7). The first group consists of EGF, TGFα, and AR. These hormones bind and activate only the EGFR, but they can activate the other three ErbB family receptors *in trans* via heterodimerization with the EGFR. The second group consists of NRG and NRG2, which bind ErbB3 and ErbB4 and transmodulate EGFR and Neu via the binding receptors. The third group consists of BTC, which binds and activates both EGFR and ErbB4. Recent data suggests that HB-EGF may also bind and activate EGFR and ErbB4, which would make HB-EGF a member of this group as well [22].

While EPR activates both EGFR and ErbB4, the interactions of EPR with these two receptors appear to be quite different. Compared to BTC, EPR stimulates higher levels of EGFR phosphorylation and lower levels of ErbB4 phosphorylation. While both EPR and BTC stimulate EGFR and ErbB4 homodimerization and signaling, the geometry of the receptor dimers induced by EPR and BTC may be subtly different; the alignment of the kinase domain of one receptor molecule of a receptor homodimer with the autophosphorylation site of the other receptor molecule following EPR stimulation could be different from this alignment following BTC stimulation, affecting the cross-phosphorylation within receptor dimers. Alternatively, ligand-induced changes in the conformation of the receptor kinase domains might be different when the receptors are activated by BTC and EPR. Therefore, BTC and EPR may differentially stimulate receptor kinase activity. In either scenario, BTC and EPR could stimulate receptor autophosphorylation on different tyrosine residues, which could also be reflected in differences in gross levels of receptor phosphorylation. In this manner BTC and EPR could differentially modulate receptor coupling to signaling effectors and physiologic responses.

Another difference between EPR and BTC is that while EGFR is much more sensitive than ErbB4 to EPR, EGFR is less sensitive than ErbB4 to BTC. This suggests that while the affinity of EPR for EGFR is higher than the affinity for ErbB4, the affinity of BTC for EGFR is lower than the affinity for ErbB4. This too suggests that BTC and EPR have distinct biological functions, even in cells with identical patterns of ErbB family receptor expression.

Another important aspect of EPR function is the observation that the sensitivity of ErbB4 for EPR and the magnitude of ErbB4 activation by EPR can be modulated by the expression of other ErbB family receptors. EGFR expression increases the sensitivity of ErbB4 for EPR, suggesting that EGFR-ErbB4 heterodimers have a higher affinity for EPR than ErbB4-ErbB4 homodimers (Figs. 4a,d; Figs.5a,c; Table 1). Of course an alternative explanation is that the increased ErbB4 sensitivity in the presence of EGFR is due solely to EPR-induced transphosphorylation of ErbB4 by EGFR.

ErbB2 also increases the sensitivity of ErbB4 for EPR (Figs. 4a-d; Table 1). Because EPR does not activate ErbB2 in cells devoid of EGFR or ErbB4 (Fig. 1; Fig 6), the mechanism for the increased sensitivity of ErbB4 for EPR may be that ErbB2-ErbB4 heterodimers have a higher affinity for EPR than do ErbB4-ErbB4 homodimers. These results resemble observations made with NRG, which does not bind to ErbB2, binds with low affinity to cells expressing ErbB3, and binds with higher affinity to cells that express both ErbB2 and ErbB3 [Sliwkowski, et al., 1994]. ErbB2 expression also increases the magnitude of ErbB4 activation by EPR.

These observations that ErbB4 activation by EPR can be influenced by EGFR or ErbB2 is consistent with existing models for receptor heterodimerization and transmodulation. It has been proposed that receptor heterodimerization is mediated through low-affinity hormone-receptor interactions and heterotypic receptor-receptor contacts, after which there is cross-phosphorylation by the receptor

kinase domains [23]. It is possible that EGFR and ErbB2 are favored over ErbB4 for dimerization with ErbB4 in the presence of EPR. Therefore, there would be greater ErbB4 (hetero)dimerization in cells expressing EGFR and ErbB4 or Neu and ErbB4 than in cells expressing ErbB4 alone. This may account for the increased sensitivity of ErbB4 for EPR in the presence of EGFR or ErbB2. It is also possible that ErbB2 is a better kinase for ErbB4 than ErbB4 itself. Consequently, ErbB2 may cross-phosphorylate more ErbB4 tyrosine residues in receptor heterodimers than ErbB4 would in receptor homodimers. Similarly, ErbB2 may phosphorylate the same tyrosine residues as ErbB4 to a greater extent than does ErbB4. Either of these last two possibilities would account for the increased tyrosine phosphorylation of ErbB4 by EPR in the presence of ErbB2.

As this work was being prepared for submission, another group reported that radiolabeled EPR can be cross-linked to EGFR and ErbB4 in human breast tumor cell lines but not to ErbB2 or ErbB3. Furthermore, EPR stimulated high levels of EGFR and ErbB4 tyrosine phosphorylation, and more modest levels of ErbB2 and ErbB3 tyrosine phosphorylation [24]. Because the cell lines used in these studies express at least two and in some cases all four ErbB family receptors, some caution must be used in interpreting these results. Nonetheless, these data are entirely consistent with our findings that EGFR and ErbB4 are the receptors for EPR.

To date there have been only a few clues to EPR function. EPR transcripts are not detected in normal adult mouse liver, kidney, brain, spleen, testis, or skeletal muscles. However, low levels of EPR transcripts are detectable in adult mouse lung, smooth muscle, and heart, while more robust EPR transcription is observed in whole embryo RNA samples from 7-day-old mouse embryos [25; T. Komurasaki, unpublished data]. This implies that EPR plays a significant role in early mammalian development but only a limited role in adult tissues.

Additional hints to EPR function arise from our data suggesting that EPR is a ligand for both EGFR and ErbB4. In most contexts EGFR activation is coupled to cellular DNA synthesis and proliferation. In contrast, there is mounting evidence that activated ErbB4 is coupled to growth inhibition, differentiation, and possibly tumor suppression. NRG, a ligand for ErbB3 and ErbB4, inhibits the proliferation and stimulates the differentiation of a number of human breast tumor cell lines [26], while NRG implants stimulate the differentiation of the mouse mammary epithelium *in vivo* [27]. BTC stimulates the differentiation of pancreatic AR42J cells into insulin-secreting cells, while EGF and TGFα do not [28]. Finally, agonistic anti-ErbB4 antibodies stimulate the differentiation of human breast tumor cell lines [29], and ErbB4 overexpression in breast cancer patients correlates with progesterone receptor expression, which is a marker for longer disease-free survival and better overall prognosis [30]. Because EPR is a ligand for both EGFR and ErbB4, EPR may act as a proliferative agent in cells expressing EGFR and may act as a differentiation agent in cells that express ErbB4.

Furthermore, because EPR activation of ErbB4 is regulated by ErbB2 and activated ErbB2 appears to couple to mitogenesis and cell proliferation, the effects of EPR on cells expressing ErbB4 may be tightly linked to a balance of ErbB4 and ErbB2 expression: in cells having relatively low levels of ErbB2, EPR may have little effect, because it fails to bind to ErbB4; in cells having moderate levels of ErbB2 and high levels of ErbB4, EPR may act as a differentiation agent and inhibit cell proliferation because the relatively high levels of ErbB4 signaling may overcome the effects of ErbB2 signaling; and finally, in cells having relatively high levels of ErbB2 relative to ErbB4, EPR may stimulate such high levels of ErbB2 signaling that the effects of ErbB4 signaling are overcome and cell proliferation is stimulated. In sum, our data suggests that the physiologic response to EPR will be dictated by relative levels of

EGFR, ErbB2, and ErbB4 expression, and not just the absolute level of expression of any single ErbB family receptor.

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Table 1

Cell Line & Receptor	[EPR] Yielding Half-maximal Receptor Activation	[BTC] Yielding Half-maximal Receptor Activation
BaF3/EGFR	380ng/ml	NT
CEM/ErbB4	1790ng/ml	NT
BaF3/EGFR+ErbB4		
EGFR:	320ng/ml	35ng/ml
ErbB4:	790ng/ml	5ng/ml
CEM/ErbB2+ErbB4		
ErbB2:	400ng/ml	NT
ErbB4:	630ng/ml	NT

Data is taken from Figures 4a-d and 5a-c.

Figure Legends

Figure 1. EPR Stimulation of Receptor Phosphorylation in BaF3 Cells Expressing a Single ErbB Family Receptor.

BaF3/EGFR, BaF3/ErbB2, and BaF3/ErbB4 cells [14] were stimulated with 200 ng/ml EPR (E), 200 ng/ml BTC (B), 188 ng/ml synthetic NRGβ (N), or were mock stimulated with phosphate-buffered saline (M) as described previously [7,14]. ErbB family receptors were immunoprecipitated from lysed cells using specific anti-receptor antibodies and separated by SDS/PAGE as previously described [7,14]. Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal anti-phosphotyrosine antibody as previously described [7,14].

Figure 2a,b. EPR Stimulation of Receptor Phosphorylation in BaF3 Cells Expressing Combinations of ErbB Family Receptors.

BaF3/EGFR+ErbB2, BaF3/EGFR+ErbB3, BaF3/EGFR+ErbB4, BaF3/ErbB2+ErbB3, and BaF3/ErbB3+ErbB4 cells [14] were stimulated with 200 ng/ml EPR (E), 200 ng/ml TGFα (T), 188 ng/ml synthetic NRGβ (N), or were mock stimulated with phosphate buffered saline (M) as described previously [7,8,14]. EGFR (α1), ErbB2 (α2), ErbB3 (α3), or ErbB4 (α4) was immunoprecipitated from lysed cells using specific anti-receptor antibodies and separated by SDS/PAGE as previously described [7,14]. Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal anti-phosphotyrosine antibody as previously described [7,14].

Figure 3. EPR Stimulation of Receptor Phosphorylation in CEM Cells Expressing Either ErbB4 Alone or Both ErbB2 and ErbB4.

CEM/ErbB4 and CEM/ErbB2+ErbB4 cells [10] were stimulated with 100 ng/ml BTC (B), 100 ng/ml recombinant NRGβ (N), 1000 ng/ml EPR (E), or were mock stimulated with phosphate-buffered saline (M) as described previously [Riese, et al., 1995]. ErbB2 (α2), or ErbB4 (α4) was immunoprecipitated from lysed cells using specific anti-receptor antibodies and separated by SDS/PAGE as previously described [7,14]. Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal anti-phosphotyrosine antibody as previously described [7,14].

Figure 4a,-d EPR Dose Response in BaF3 Cells Expressing EGFR or in CEM Cells Expressing ErbB4 alone or both ErbB2 and ErbB4.

(4a,b) BaF3/EGFR, CEM/ErbB4, or CEM/ErbB2+ErbB4 cells were stimulated with 100 ng/ml BTC (BTC), increasing concentrations of Epiregulin as indicated, or were mock stimulated with phosphate-buffered saline (Mock) as described previously [7,14]. EGFR, ErbB2, or ErbB4 was immunoprecipitated as indicated or appropriate using specific anti-receptor antibodies and separated by SDS/PAGE as previously described [Riese, et al., 1995]. Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal anti-phosphotyrosine antibody as previously described [7,14].

(4c,d) Anti-phosphotyrosine immunoblot images were scanned on a Hewlett-Packard ScanJet 3p flatbed scanner set for 600dpi optical resolution. Images were cropped using Adobe Photoshop and receptor tyrosine phosphorylation was quantified using NIH Image. Net receptor tyrosine phosphorylation was calculated by subtracting the receptor tyrosine phosphorylation exhibited by mock stimulated cells. Tyrosine phosphorylation either was expressed relative to the tyrosine phosphorylation stimulated by 100 ng/ml BTC (**4c**) or was expressed relative to the maximal receptor tyrosine phosphorylation stimulated by EPR (**4d**).

Figure 5a,-c EPR and BTC Dose Response in BaF3 Cells Expressing both EGFR and ErbB4.

(5a) BaF3/EGFR+ErbB4 cells were stimulated with increasing concentrations of Betacellulin or Epiregulin or were mock stimulated with phosphate-buffered saline (Mock) as described previously [7,14]. EGFR or ErbB4 was immunoprecipitated as indicated using specific anti-receptor antibodies and separated by SDS/PAGE as previously described [7,14]. Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal anti-phosphotyrosine antibody as previously described [7,14].

(5b,c) Anti-phosphotyrosine immunoblot images were scanned on a Hewlett-Packard ScanJet 3p flatbed scanner set for 600dpi optical resolution. Images were cropped using Adobe Photoshop and receptor tyrosine phosphorylation was quantified using NIH Image. Net receptor tyrosine phosphorylation was calculated by subtracting the receptor tyrosine phosphorylation exhibited by mock stimulated cells. Tyrosine phosphorylation stimulated by BTC (**5b**) or EPR (**5c**) was expressed relative to the maximal tyrosine phosphorylation stimulated by BTC.

Figure 6. EPR Stimulation of IL3-independent Responses in BaF3 Cells Expressing Various ErbB Family Receptors.

The IL3 independent responses of BaF3/LXSN (vector control), BaF3/EGFR, BaF3/ErbB2, and BaF3/ErbB2+ErbB4 cells to EPR stimulation were assayed as described earlier [7,14]. Briefly, cells were seeded in duplicate or triplicate at an initial density of $100x10^3$ cells/ml in medium lacking IL3 (IL3-Free), containing IL3 (IL3), or lacking IL3 but supplemented with 10 ng/ml EPR (Epiregulin), 10 ng/ml BTC (Betacellulin), 10ng/ml synthetic NRGβ (Neuregulin) or 10 ng/ml TGFα (TGF-alpha). After seeding, samples were taken every 24 hours and the viable cell density was calculated by staining cells with trypan blue and counting them in a hemacytometer. Samples were taken until the viable cells reached a saturation density. The mean and standard error densities for three to seven trials are shown. "NT" indicates not tested.

Figure 7. Venn Diagram Illustrating the Activities of EGF Family Hormones.

The three functional groups of EGF family hormones are illustrated using a Venn diagram. The diagram is based on data presented in this work and data from references 7, 8, 14, and 22.

Figure 1

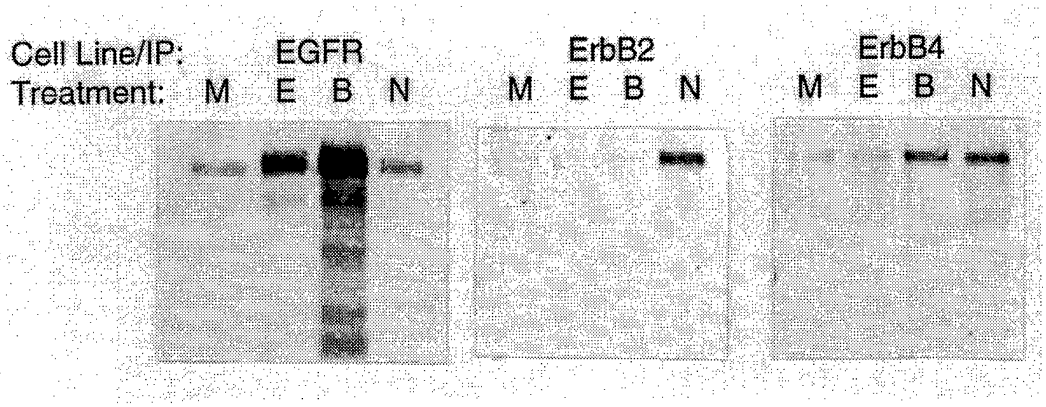


Figure 2a

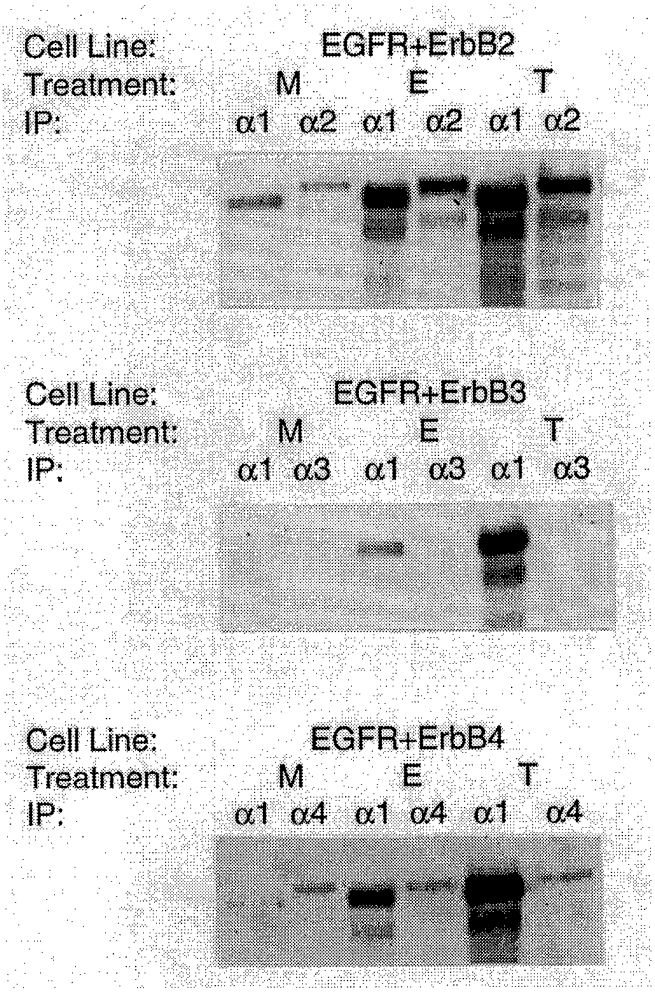


Figure 2b

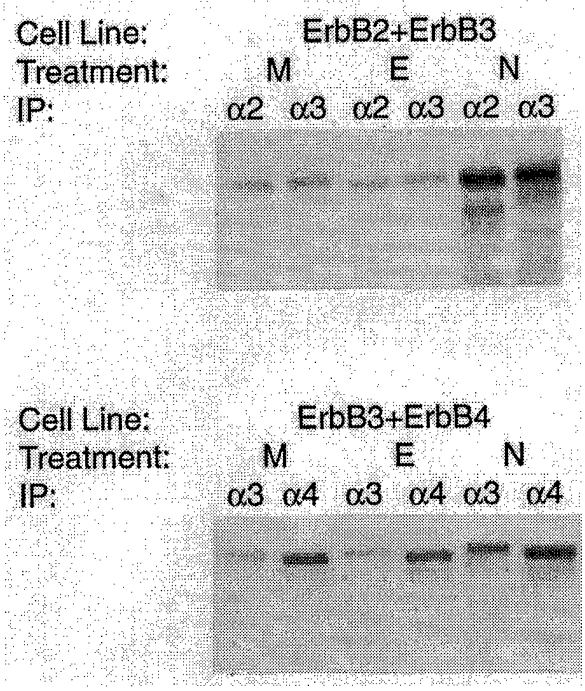
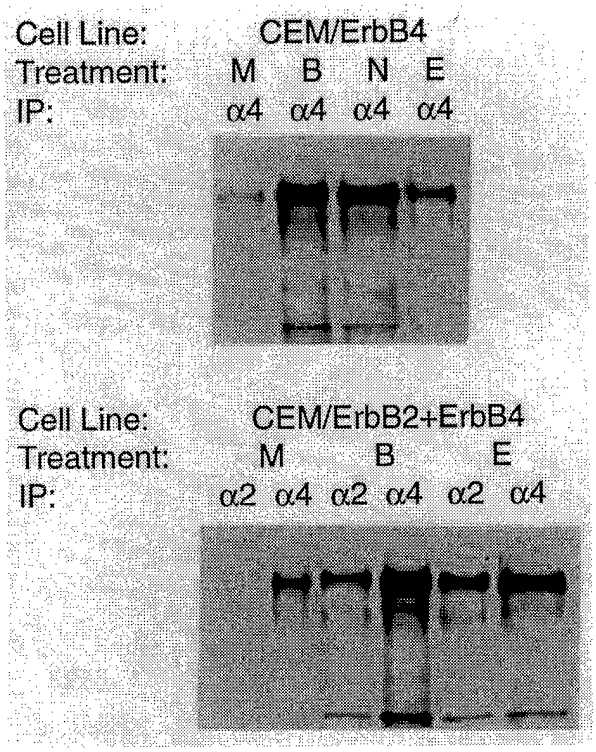


Figure 3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CEM/ErbB4
Treatment:
M B N E
IP:
α4 α4 α4 α4
Cell Line:
CEM/ErbB2+ErbB4
Treatment:
M B E
IP:
α2 α4 α2 α4 α2 α4

Figure 4a

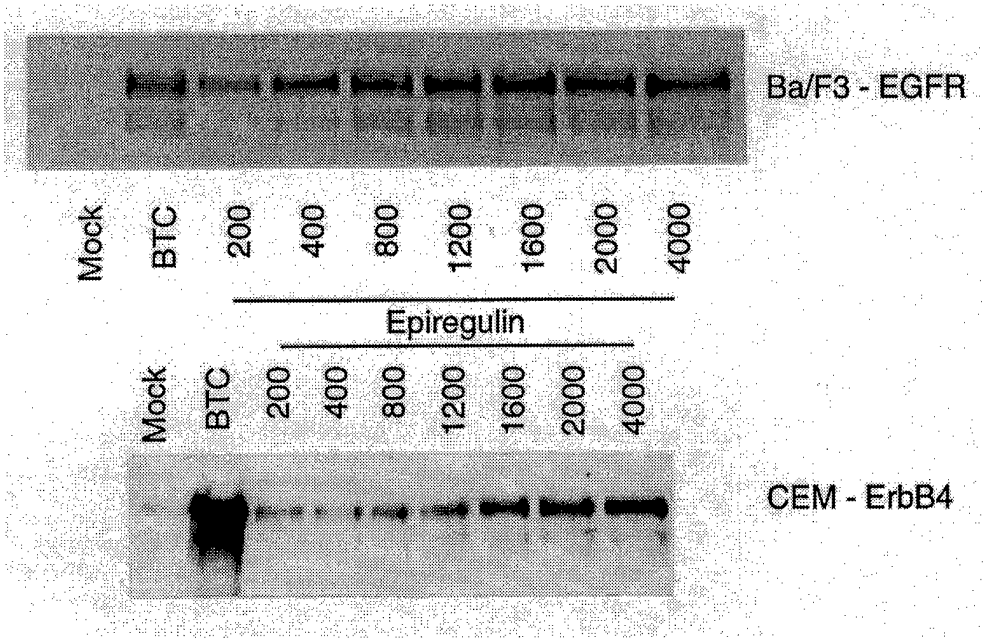


Figure 4b

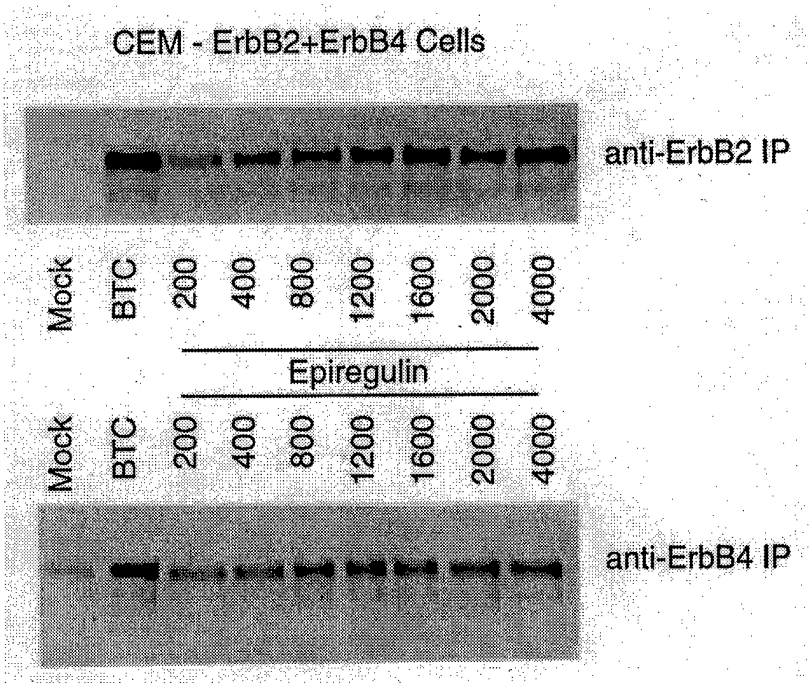


Figure 4c

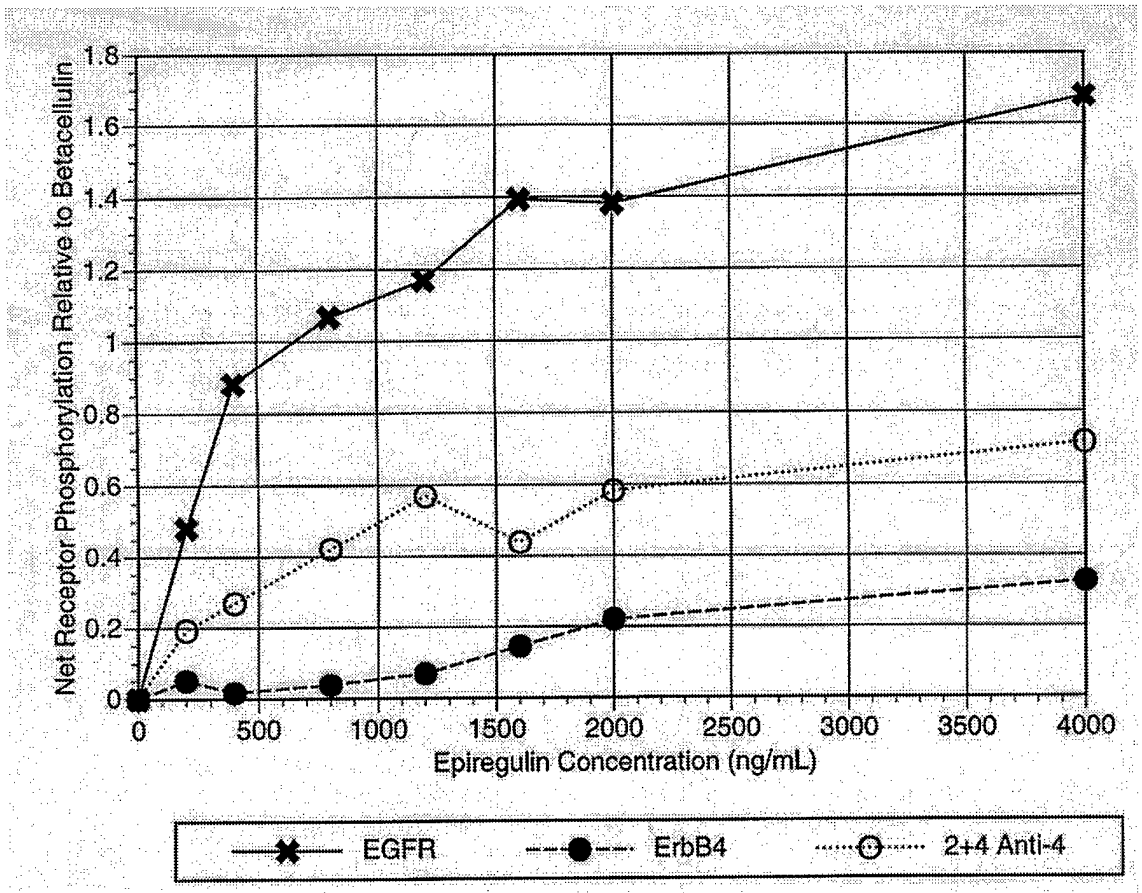


Figure 4d

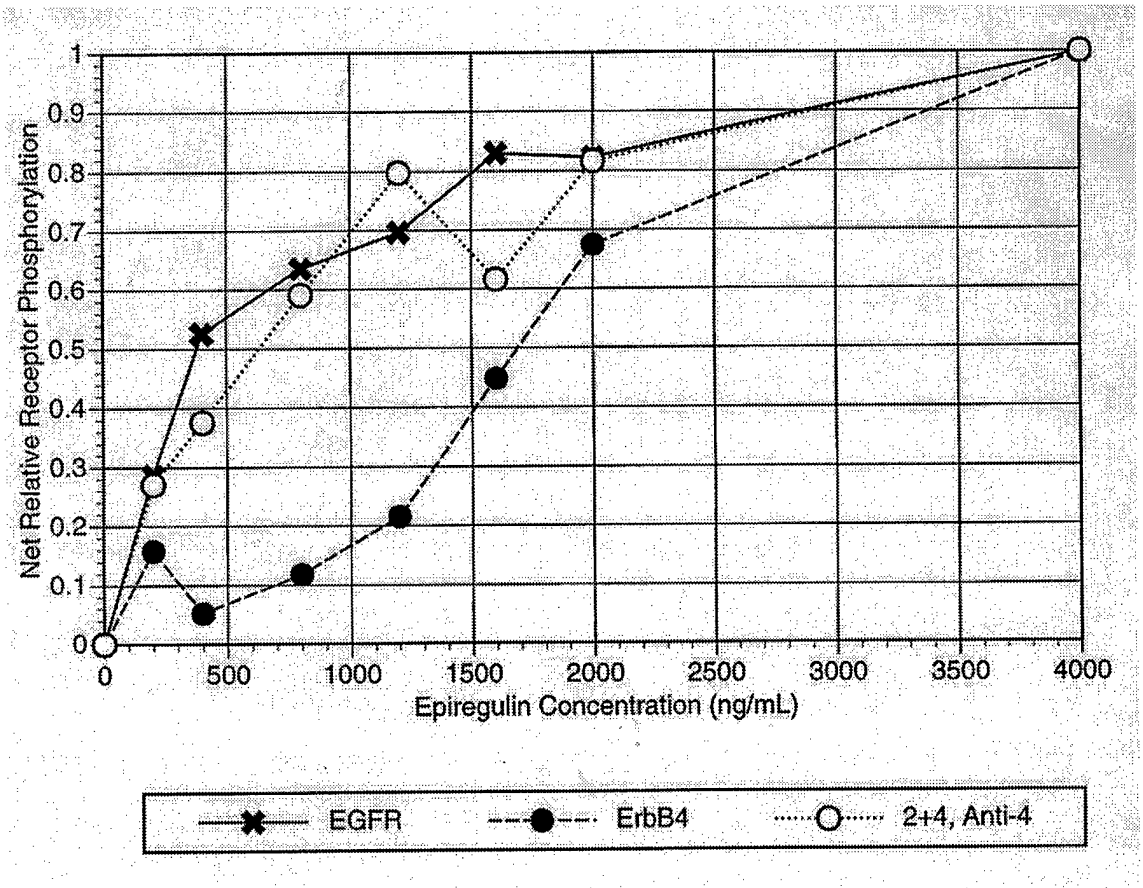


Figure 5a

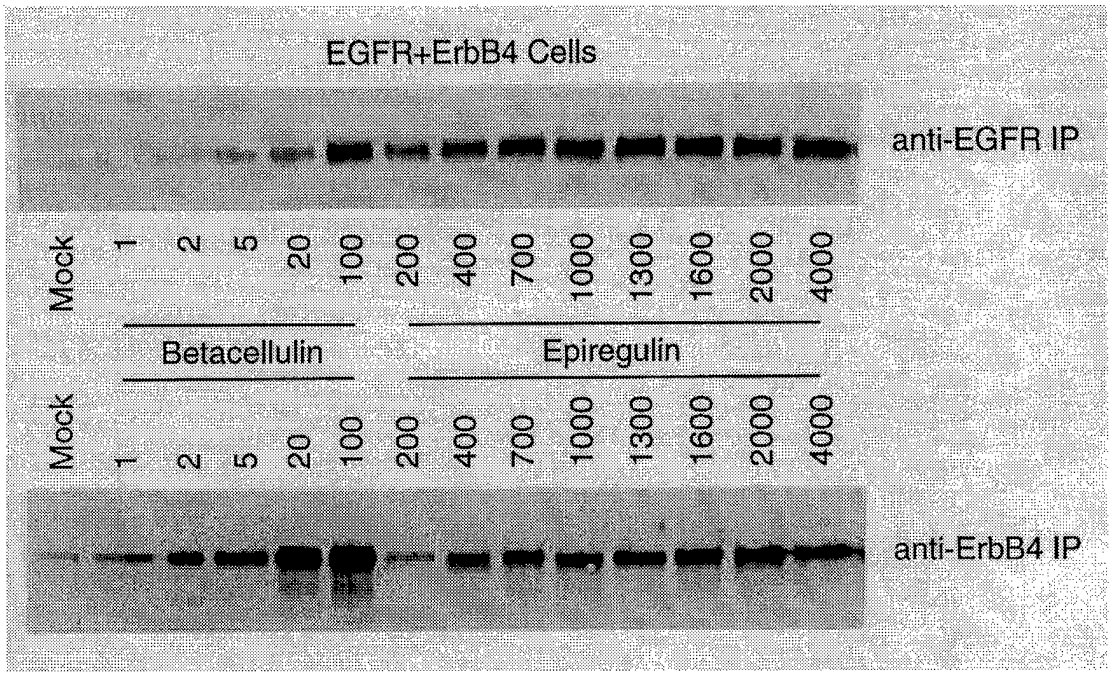

EGFR+ErbB4 Cells
anti-EGFR IP
Mock
1
2
5
20
100
200
400
700
1000
1300
1600
2000
4000
Betacellulin
Epiregulin
Mock
1
2
5
20
100
200
400
700
1000
1300
1600
2000
4000
anti-ErbB4 IP

Figure 5b

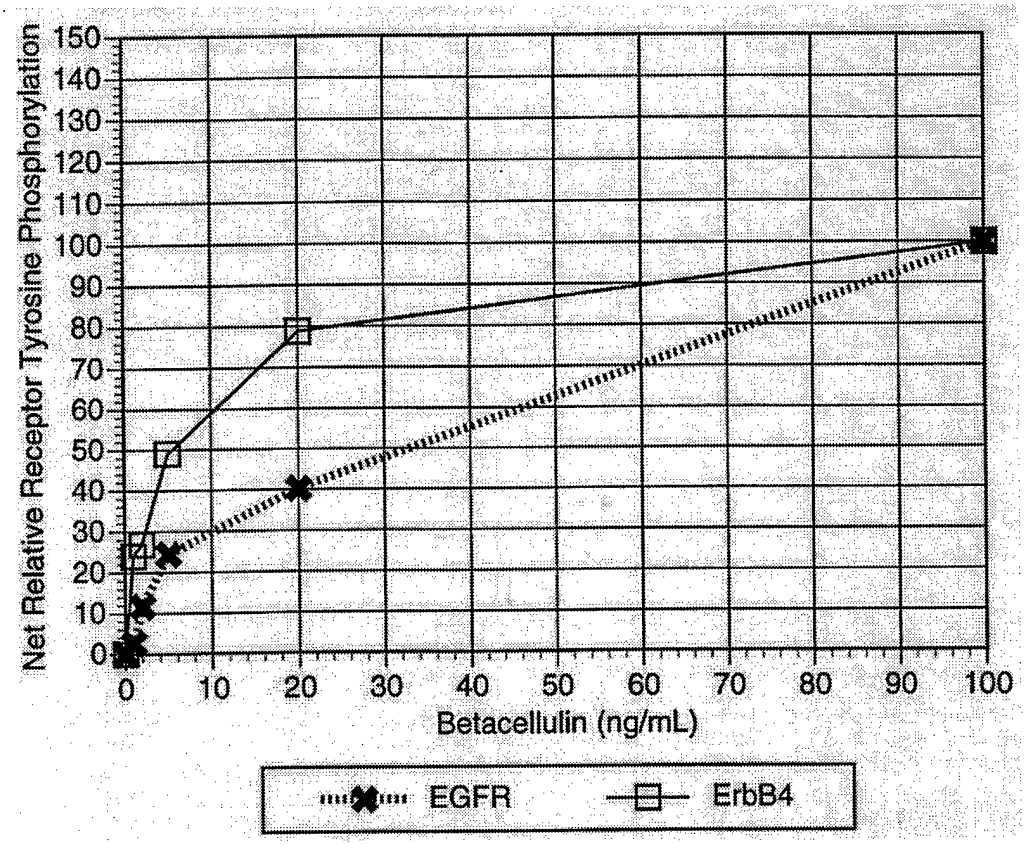


Figure 5c

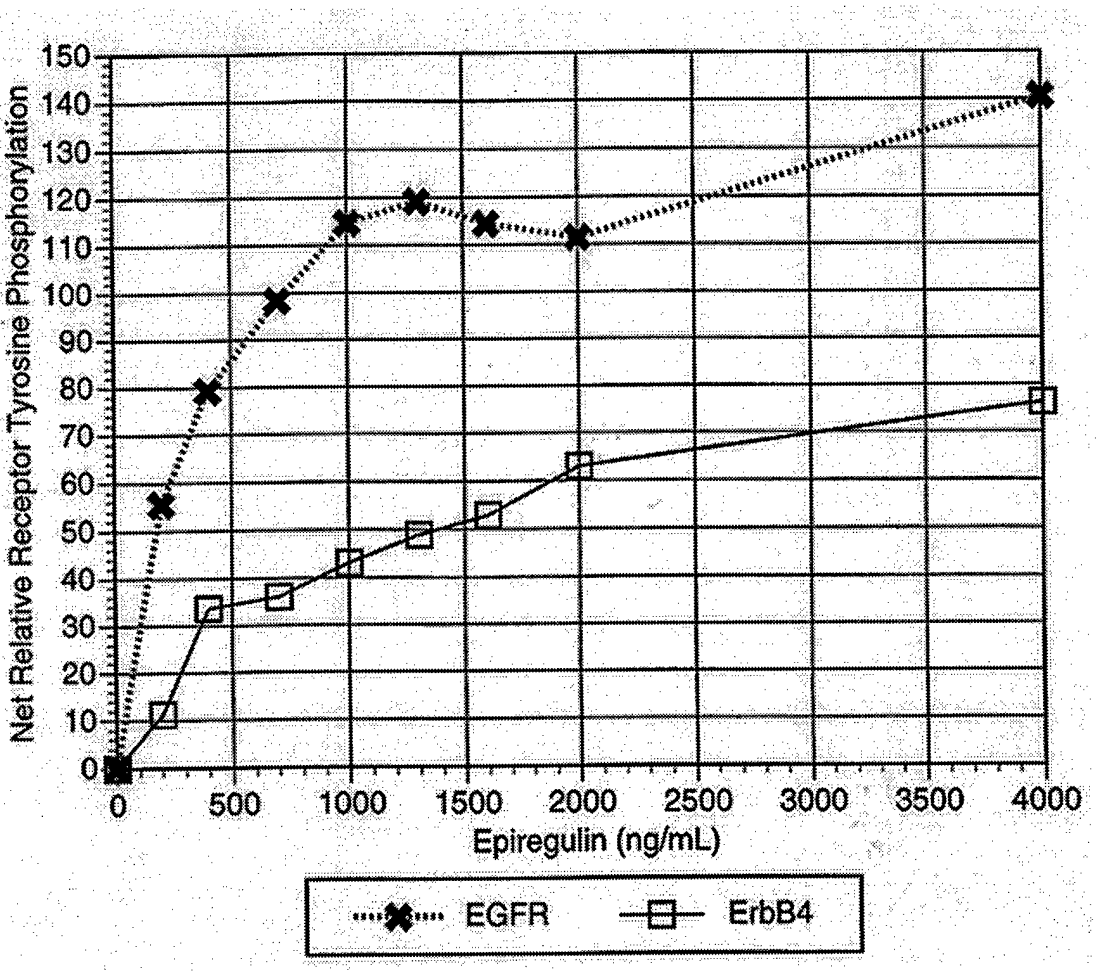


Figure 6

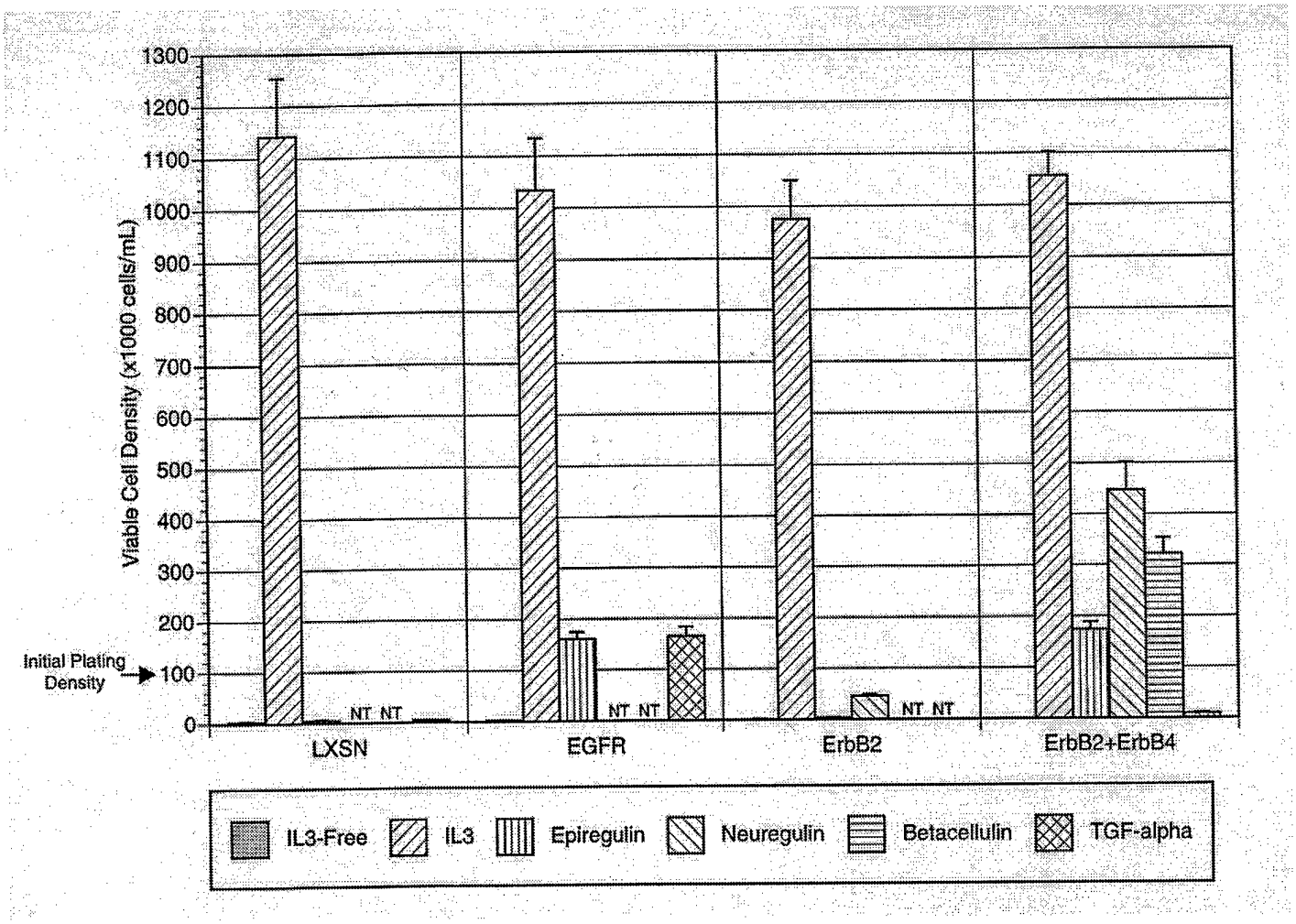
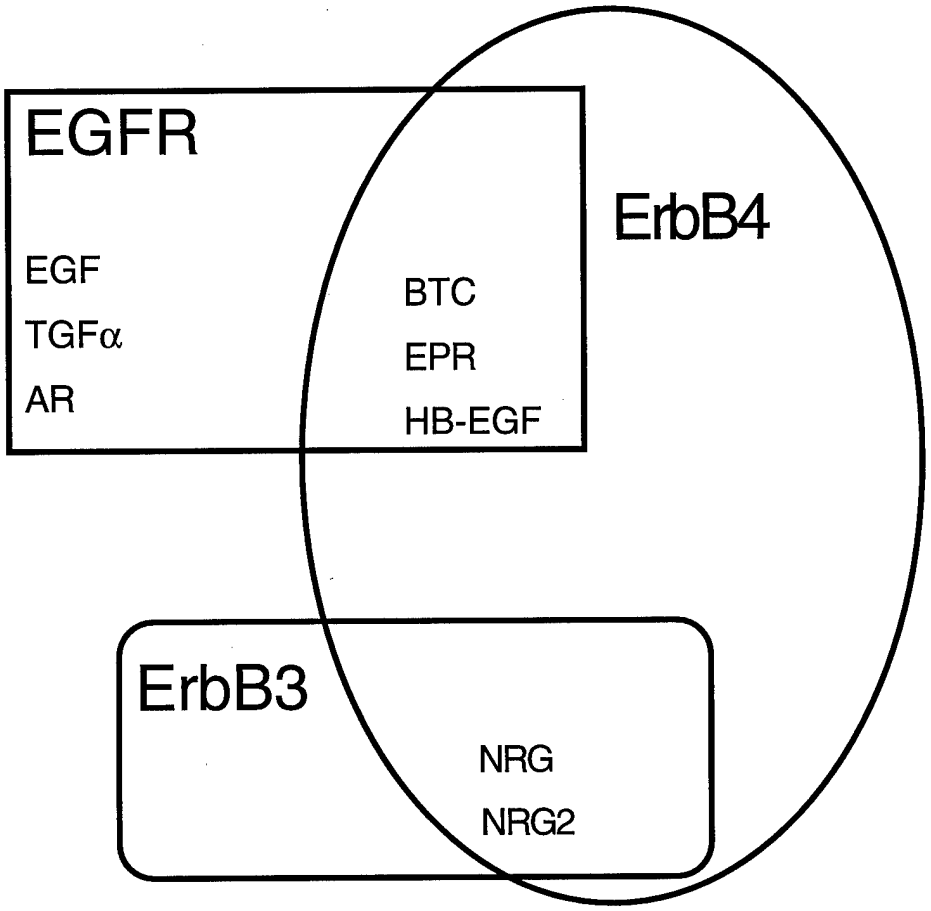

Viable Cell Density (x1000 cells/mL)
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Initial Plating Density
NT NT
NT NT
NT NT
LXSN
EGFR
ErbB2
ErbB2+ErbB4
IL3-Free
IL3
Epiregulin
Neuregulin
Betacellulin
TGF-alpha

Figure 7



Specificity Within the EGF Family/ErbB Receptor Family Signaling Network

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I. Summmary

Recent years have witnessed tremendous growth in the epidermal growth factor (EGF) family of peptide growth factors and the ErbB family of tyrosine kinases, the receptors for these factors. Accompanying this growth has been an increased appreciation for the roles these molecules play in tumorigenesis and in regulating cell proliferation and differentiation during development. Consequently, a significant question has been how diverse biological responses are specified by these hormones and receptors. Here we discuss several characteristics of hormone-receptor interactions and receptor coupling that contribute to specificity: (1) A single EGF family hormone can bind multiple receptors; (2) A single ErbB family receptor can bind multiple hormones; (3) There are three distinct functional groups of EGF family hormones; (4) EGF family hormones can activate receptors in *trans* and this heterodimerization diversifies biological responses; (5) ErbB3 requires a receptor partner for signaling; and (6) ErbB family receptors differentially couple to signaling pathways and biological responses.

II. Introduction

The signaling network composed of the epidermal growth factor (EGF) family of hormones and their receptors regulates the proliferation and differentiation of many tissue types. Deregulation of this network is a significant factor in the genesis or progression of several human cancers, including neoplasms of the brain, lung, breast, ovary, pancreas and prostate[1,2]. These observations have spurred efforts to elucidate how this signaling network is regulated and coupled to physiologic responses, and how regulation and coupling are disrupted in malignancies.

Efforts to characterize this signaling network have also been triggered by the observations that the EGF family peptides called neuregulins (NRGs), play a significant role in neural development and function. Neurons produce NRGs, while post-synaptic cells or cells associated with neurons (glia or Schwann cell precursors) express ErbB family receptors. NRG activates these receptors through a paracrine or juxtacrine mechanism. For example, NRGs produced by motor neurons induce acetylcholine receptor subunit transcription and protein synthesis in post-synaptic muscle cells, which express ErbB2 and ErbB3 and possibly EGFR and ErbB4[3]. Furthermore, NRGs expressed from sensory neuron axons stimulate Schwann cell proliferation and may promote the differentiation of neural crest cells into Schwann cell precursors[4]. In general, patterns of ErbB3 and ErbB4 expression differ during neural development, suggesting that these NRG receptors couple to distinct signaling pathways and cellular responses[3]. Additional functions for NRGs have been identified in mutants in which NRG signaling is disrupted[5-8]. Such mutants display defects in the peripheral nervous system, most notably a loss of cells in the cranial sensory ganglia or a mis-innervation of rhombomeres by cranial sensory and motor neurons. Again, because multiple ErbB family receptors appear to be NRG effectors, there is

significant interest in how these receptors couple to distinct signaling effectors and biological function.

A. EGF Family Hormones

There at least eight different hormones in the EGF family: EGF itself, Transforming Growth Factor alpha (TGFα); Heparin-binding Epidermal Growth Factor-like Factor (HB-EGF); Amphiregulin (AR), also known as Keratinocyte Autocrine Factor or Colorectum-Cell Derived Growth Factor; Epiregulin (EPR); Betacellulin (BTC); the Neuregulins (NRGs), also known as Heregulins, Neu Differentiation Factors, Glial Growth Factors, Acetylcholine Receptor Inducing Activity, or Sensory and Motor Neuron-Derived Factor; and the Neuregulin2s (NRG-2s), also known as the Cerebellum-Derived Growth Factors. Moreover, multiple NRG and NRG-2 isoforms arise from alternative transcriptional splicing[1-4,9-11].

Most EGF family peptides are synthesized as transmembrane precursors that can be proteolytically cleaved to release the soluble form of the hormone, or can function as membrane-anchored hormones in juxtacrine signaling. The soluble hormones can be as small as 50 amino acids, sharing a domain of homology that encompasses approximately 50 amino acids. The salient feature of this domain is six characteristically-spaced cysteine residues that form three intramolecular disulfide linkages and define a three-loop secondary structure[2]. This domain is both required and sufficient for ErbB family receptor binding and activation; little is known about the physiological functions of the non-EGF homologous domains, which can be extensive.

Two additional proteins share limited homology with EGF family hormones. Cripto-1[12,13] and Cryptic[14] contain the six cysteine residues

characteristic of EGF family hormones. However, the spacing of these residues is altered such that Cripto-1, and Cryptic completely lack the "A-loop" formed by the residues between the first and second cysteine residues, and the "B-loop" formed by the residues between the third and fourth cysteine residues is considerably smaller than this domain in other EGF family hormones. Indeed, the synthetic EGF homology domain of Cripto-1 does not activate ErbB family receptors[15].

B. ErbB Family Receptors

There are four ErbB family receptors: Epidermal Growth Factor Receptor (EGFR - also called HER; ErbB)[16]; ErbB2 (also Neu, HER-2)[17,18]; ErbB3 (HER3)[19,20]; and ErbB4 (HER4)[21]. The human forms of these receptors range in size from 1210 to 1343 amino acids. They each consist of a cysteine-rich extracellular domain, a single membrane-spanning domain, and a large cytoplasmic domain composed of a tyrosine kinase domain and several tyrosine residues that are phosphorylated upon receptor activation. Ligand binding stimulates receptor dimerization and tyrosine phosphorylation at several sites that then serve to dock effector proteins and couple to physiologic responses.

III. Differential Activation and Coupling of ErbB Family Receptors

A. Differential Activation of ErbB Family Receptors

A number of mechanisms contribute to the complexity and inter-connectedness of the EGF family/ErbB family signaling network. These include the large number of ligands and extensive cross-interactions among the receptors.

1. A single EGF family hormone can bind multiple receptors. For example, BTC, HB-EGF, and EPR activate both the EGF receptor and ErbB4, while NRG and NRG-2 both bind ErbB3 and ErbB4 (Fig. 1).

2. A single ErbB family receptor can bind multiple hormones. With the exception of ErbB2, which is an orphan receptor, each ErbB family receptor binds multiple hormones. EGF, TGFα, HB-EGF, AR, BTC, and EPR bind to the EGFR. BTC, NRG, NRG-2, HB-EGF, and EPR bind ErbB4, but only NRG and NRG-2 bind ErbB3 (Fig. 1).

3. There are three distinct functional groups of EGF family hormones. These groups are distinguished by their abilities to bind to and activate distinct sets of individual receptors (Fig. 1). The first group consists of EGF and its functional analogs TGFα, and AR, which all bind and activate EGFR but not the other receptors. The second group consists of NRG and NRG-2, which bind ErbB3 and ErbB4. The third group consists of BTC, EPR, and HB-EGF. These hormones bind and activate both EGFR and ErbB4.

4. EGF family hormones can activate receptors in trans. Receptors that do not bind a particular hormone when expressed alone can be cross-activated ("transmodulated") if a binding-competent receptor is also present. For example, although EGF does not bind to or activate ErbB2 expressed by itself, EGF induces the tyrosine phosphorylation of both EGFR and ErbB2 in cells expressing both

receptors[22-24] (Figure 1). Furthermore, even a kinase-inactive ErbB2 mutant can be cross-activated by EGF and EGFR[25]. Since the transmodulation of ErbB2 by the EGFR is accompanied by the formation of EGF-stimulated EGFR-ErbB2 heterodimers[26-27], it is likely that the kinase responsible for ErbB2 transmodulation is the EGFR itself. Nonetheless, a plausible alternative is that a *src*-family kinase activated by the EGFR is involved[28].

Analogous heterotypic interactions are now known to occur extensively among other combinations of ErbB family receptors (Fig. 1). The presence of a single hormone-binding receptor is generally sufficient for EGF family hormones to activate all other ErbB family members present. However, RTKs outside the ErbB receptor family do not cross-activate these receptors, nor can they themselves be activated *in trans* by EGF family hormones and ErbB family receptors. There are two notable exceptions: While BTC activates ErbB4, BTC does not activate ErbB3 in cells expressing both ErbB3 and ErbB4 (Fig. 1)[29]. Similarly, while NRGα binds ErbB3, it does not activate either EGFR or ErbB3 in cells expressing these receptors[30].

At a more quantitative level, there is a graded hierarchy of heteromeric interactions that may reflect differences in affinities of the various hormone-receptor-receptor complexes. For example, EGF, which binds only the EGFR, transmodulates ErbB2 more strongly than ErbB3 or ErbB4. Similarly, NRGβ, which binds ErbB3 and ErbB4, transmodulates ErbB2 more strongly than EGFR. This suggests that ErbB2 is the preferred target for transmodulation by a ligand-activated ErbB family receptor[31-33].

Since many cell types express at least three of the four receptors, this implies that there is competition among receptors for dimerization partners. This may explain the finding that pretreatment with NRG inhibits subsequent EGF binding, suggesting that recruitment of the EGFR into complexes with ErbB3

or ErbB4 reduces the availability of free EGFR[34]. Moreover, transmodulation of the EGFR in T47D cells by NRG (via ErbB3 and ErbB4) is enhanced when ErbB2 is selectively removed[33]. However, a simple competition model does not account for the observation that downregulation of ErbB2 *reduces* ErbB4 transmodulation by EGF (and the EGFR)[33].

The mechanism and stoichiometry of heteromeric receptor interactions have not been determined, and reflects the absence of a basic understanding about receptor oligomerization. For example, it is possible that higher order oligomers rather than dimers are the active signaling species. Furthermore, it is not known if a ligand binds to one or both receptors in a heterotypic receptor complex. Co-expression of binding and non-binding ErbB family members in some cases enhances hormone binding affinity[35], while intracellular retention of ErbB2 reduces EGF and NRG binding by accelerating their dissociation[36]. (We use "binding" and "non-binding" here to denote the behavior of the receptors when expressed individually.) The means by which a non-binding receptor is recruited into a receptor complex and can modulate ligand binding affinity are uncertain. Hypothetically, in EGFR/ErbB2 transmodulation, EGF binding may unveil a cryptic EGFR/ErbB2 inter-receptor binding site, and/or may stabilize EGF binding to ErbB2. Similarly, formation of EGFR-ErbB2 dimers may alter the conformation of the ErbB2 hormone binding domain or create a composite binding site encompassing elements from both receptors. EGF appears to bind the EGFR with a 1:1 stoichiometry[37,38] and it has been proposed that EGF binds bivalently, with each EGF molecule binding to a high affinity site on one EGFR molecule and a low affinity site on another EGFR molecule[38]. Bivalent hormone binding is consistent with the observation that BTC and EGF-NRG chimeras bind both EGFR and ErbB4[29,39]. It has been proposed that hormone binding to the high affinity binding site is required to stabilize receptor dimers

and for receptor activation. Therefore, bivalent binding of a hormone molecule to a high affinity site on one receptor ("binding receptor) and a lower affinity site on a heterotypic ErbB family receptor molecule ("non-binding" receptor) may be the mechanism underlying receptor heterodimerization and transmodulation. Differences in the affinity of this "lower affinity" binding site on the "non-binding" receptor may explain the variable heterodimerization and transmodulation potential for the four ErbB family receptors. And finally, these possibilities mean that co-expression of ErbB family receptors may create novel "emergent" binding specficities.

Intriguing observations that have led to a greater understanding of ErbB family receptor heterodimerization are that NRG induces the formation of EGFR-Neu heterodimers and that this heterodimerization is blocked by the Neu tyrosine kinase inhibitor tyrphostin AG879. Ligand-induced ErbB family receptor dimers apparently dissociate and can nucleate "secondary" (hetero)dimerization with additional receptor molecules. Moreover, receptor phosphorylation is apparently required for dissociation of the "primary" receptor dimers and for formation of "secondary" heterodimers[40]. Because receptor phosphorylation also triggers receptor internalization and degradation, the amount of receptor heterodimerization must be regulated by a number of factors, including hormone and receptor concentration, the affinity for receptor dimerization, receptor kinase activity, and rate of receptor internalization.

5. *ErbB3 requires a partner.* A special case is the reliance of ErbB3 signaling on heteromeric interactions. Four residues in the ErbB3 tyrosine kinase homology domain diverge from the consensus tyrosine kinase sequence[20,41], and ErbB3 has little or no associated kinase activity[41]. Although ErbB3 expressed alone binds NRGs, tyrosine phosphorylation of this receptor only occurs in the

presence of additional ErbB family members. Presumably, they are required for cross-phosphorylation of ErbB3[29,35,36,42,43].

B. Coupling of ErbB Family Receptors to Biological Responses and Signaling Effectors

The complex regulation of ErbB family receptor activation is significant because the four receptors couple to distinct sets of signaling effectors and biological responses.

1. ErbB family receptors have different signaling specificities. Each receptor has unique signaling specificities, as first suggested by the unique hormone-independent transforming activity of overexpressed ErbB2 relative to the other receptors[44-51]. These differences reflect differential activation of signaling pathways, as was first shown to occur for EGFR and ErbB2[46,52].

2. ErbB family receptor heterodimerization diversifies biological responses. An early indication that activation of multiple ErbB family receptors increases response diversity was the finding that EGFR and ErbB2 synergize in transformation of NR6 fibroblasts[53]. Similarly, a subset of receptor combinations promotes hormone-independent or NRG-dependent transformation of NIH3T3 cells[48,49,51]. Interleukin 3 (IL3) -independent survival or proliferation of a panel of Ba/F3 cells with defined receptor content depends upon the specific combinations of receptors activated (Fig. 2). In this cell background, activation of ErbB4 alone yields no measurable response while activated EGFR couples to IL3-independent survival. However, activation of EGFR and ErbB4 together leads to IL3-independent proliferation[29,43]. Conversely, selective intracellular retention (and inactivation) of ErbB2 in T47D cells radically diminishes the extent and duration of MAP kinase activation by

EGF and NRG, suggesting that MAP kinase is predominantly coupled to ErbB2 activated by transmodulation[36].

The simplest interpretation for receptor cooperativity in induction of biological responses is that different receptors activate complementary signaling pathways. However, an alternative explanation is that receptor heteromers have unique signaling specificities. This is plausible, since in a cross-phosphorylation reaction within a heterodimer, the identity and geometry of kinase/substrate pairs differ from that for a homodimer. In NIH3T3 cells ectopically expressing EGFR and ErbB3, NRG stimulates anchorage-independent growth, while EGF does not, despite the fact that EGF stimulates higher levels of receptor phosphorylation than NRG. Furthermore, EGF treatment mobilizes intracellular Ca^{2+} stores, while NRG treatment does not[51]. Similarly, when the EGFR is activated by HB-EGF in a breast tumor cell line, the downstream signaling effector Cbl is tyrosine phosphorylated and complexes with the EGFR. However, when EGFR is activated by NRG through transmodulation by ErbB3 or ErbB4, Cbl is not tyrosine phosphorylated nor does it complex with the EGFR, despite the fact that HB-EGF and NRG stimulate similar levels of EGFR phosphorylation[33]. These data suggest that distinct sets of receptor tyrosine residues become phosphorylated in response to different stimuli, resulting in differential coupling to signaling effectors and biological responses.

3. ErbB family receptors couple to distinct cellular signaling effectors. Although it is clear that ErbB family receptors differentially couple to biological responses the specific substrates and pathways associated with these different responses have not been elucidated. A number of receptor effectors have been identified, a few of which may be receptor-specific[1,31,36,46,52,54-59]. For example, Cbl is activated by and complexes with the EGFR but not the other ErbB family

receptors[60]. Similarly, the CHK Csk-homologous kinase binds to ErbB2, but does not bind EGFR, ErbB3, or ErbB4[61].

IV. Conclusions

Emerging evidence demonstrates that the extensive cross-interactions revealed by tissue culture analysis of the EGF system has profound importance *in vivo*. This is manifested by the striking phenotypic similarities of mice with homozygous disruptions of the ErbB2, ErbB4, and NRG genes[5-8]. These three gene disruptions are all embryonically lethal at day 10.5 post coitum and induce overlapping but not identical defects in the nervous system. Significantly, these animals all lack the trabecular extensions of the ventricular myocardium, which results in lethality due to cardiac malfunction. ErbB2 and ErbB4 are expressed in the myocardium, while NRG is expressed in the endocardium. The disruption phenotypes show that paracrine activation of both ErbB2 and ErbB4 by NRG is required for proper myocardial development and verifies the importance of transmodulation of ErbB2 in a physiologic response to NRG[4,62].

A number of open questions regarding the regulation and coupling of this signaling network remain. For the hormones, there is little understanding of the role of the non-EGF homologous domains, which can be quite large and contain a number of recognizable motifs. For example, some of the differentially-spliced NRG isoforms include an immunoglobulin (Ig)-like domain, a glycosylation-rich spacer domain, a cysteine-rich "sensory and motor neuron-derived factor" domain, and a variant kringle domain[9,63]. While these domains are not required for activation of ErbB3 or ErbB4, they may regulate hormone-receptor interactions in a quantitative manner. In fact, mutant mice homozygous for a mutant NRG gene containing a disruption in the Ig-like domain die during embryogenesis and exhibit neurologic and cardiac defects

similar to those observed in mice homozygous for complete disruption of the NRG gene[5,8]. While this implies that the Ig-like domain is required for ErbB family receptor activation, another possibility is that this domain is required for NRG stabilization or presentation by extracellular matrix *in vivo*. In a similar vein, only a little more is known about regulation of hormone-receptor interactions by heparin-sulfate proteoglycans (HSPGs). NRG, HB-EGF, and AR bind HSPGs, and HSPGs regulate the interactions of HB-EGF and AR with the EGFR[64]. HSPGs may regulate ligand binding by acting as low affinity receptors for AR and HB-EGF, increasing the local concentration of these hormones.

EGF family hormones are initially synthesized as membrane-anchored precursors that are subsequently cleaved to release soluble hormone. Experiments using mice homozygous for disruptions in the ErbB4 and NRG genes suggest that activation of ErbB family receptors by membrane-anchored hormones on an adjacent cell or tissue (juxtacrine regulation) is required for some developmental processes. It is unclear, however, whether the membrane-anchored and soluble forms of EGF family hormones stimulate identical patterns of receptor tyrosine phosphorylation. Steric and conformational constraints on the membrane-anchored hormones might restrict their activities. Several of the membrane-anchored hormone precursors possess significant cytoplasmic tails. During juxtracrine receptor activation the membrane-anchored hormones may also act as receptors, coupling to signaling effectors and physiologic changes in response to ErbB family receptor binding. This possibility has stimulated efforts to identify proteins that interact with the cytoplasmic domains of EGF family prohormones. The cytoplasmic domain of TGFα has been shown to associate with an uncharacterized protein kinase activity as well as a p86 cytoplasmic protein. However, the identities of the protein kinase and the p86 cytoplasmic

protein have yet to be determined and the regulation of these interactions has yet to be defined[65-66].

Another emerging field of study is the role of ErbB family receptors as effectors for stimuli independent of EGF family hormones [67], much as non-receptor protein tyrosine kinases serve as signaling effectors for receptors lacking kinase activity. Treatment of HeLa cells with short-wavelength ultraviolet light (UVC) stimulates EGFR and p42 MAP kinase phosphorylation and induces c-*fos* and c-*jun* transcription. These responses are not seen in HeLa cells ectopically expressing a dominant-negative EGFR mutant[68]. It has been proposed that one mechanism by which UV induces cellular responses is through the induction of reactive oxygen species (ROS), such as hydrogen peroxide. Therefore, it is not surprising that in vascular smooth muscle cells hydrogen peroxide also stimulates the phosphorylation of EGFR and MAP kinases as well as the formation of complexes containing Shc, Grb2, SOS, and EGFR[69]. Surprisingly, EGF stimulates hydrogen peroxide generation in A431 human carcinoma cells, and eliminating hydrogen peroxide with catalase reduces EGFR tyrosine phosphorylation in response to EGF. It has been proposed that hydrogen peroxide inhibits a protein phosphatase specific for the EGFR and that this inhibition is required for the maintenance of EGFR in its phosphorylated state[70]. Therefore, cellular stress, which in some cases is accompanied by ROS release, may be coupled to the MAP kinase signaling pathway through EGFR and perhaps other ErbB family receptors as well.

There is also increasing evidence that G-protein-coupled serpentine receptors (GPCR) also regulate ErbB family receptor signaling. The GPCR ligands thrombin, endothelin-1, and lysophosphatidic acid all activate Neu, EGFR, Shc, Grb2, and the ERK1 and ERK2 MAP kinases in Rat-1 fibroblasts. Furthermore, activation of these signaling pathways by GPCR ligands is disrupted in Rat-1 cells

expressing a dominant negative EGFR mutant or in Rat-1 cells pre-incubated with the EGFR antagonist tyrphostin AG1478[71]. The mechanism for ErbB family receptor phosphorylation in response to GPCR ligands is still unclear, although it has been proposed that src family kinases may be involved. An interesting twist is that the purified EGFR phosphorylates the $G_{s\alpha}$ subunit of the heterotrimeric G protein complex *in vitro* and that phosphorylated $G_{s\alpha}$ displays increased GTPase activity and greater GTPγS binding capacity and that phospho-$G_{s\alpha}$ augmented adenyl cyclase activity in S49 cyc^- cell membranes[72-73]. Since EGF stimulates cAMP accumulation in the heart via $G_{s\alpha}$[72] cross-talk between ErbB family receptors and the GPCR signal transduction pathway may be bidirectional.

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Figure Legends

Figure 1. Three distinct functional groups of EGF family hormones.

The patterns of ErbB family receptor activation stimulated by various EGF family hormones are shown for cell lines expressing ErbB family receptors singly or in pairwise combinations. Where there are quantitative variations within groups, the pattern shown applies to the prototype member listed first. A filled box indicates receptor activation, with the fill darkness indicating the intensity of receptor activation. A white "x" on a filled background indicates that the hormone binds the receptor but does not activate it. Adapted from references 10, 29, 31, 35, 36, 42, 43, 74-83.

Figure 2 - Responses of Recombinant Ba/F3 Cell Lines to ErbB Family Receptor Activation

The patterns of interleukin-3 (IL3) -independent responses stimulated by various EGF family hormones are shown for Ba/F3 cell lines expressing ErbB family receptors singly or in pairwise combinations. A filled box indicates an IL3-independent response, with a gray fill indicating IL3-independent survival and a black fill indicating IL3-independent proliferation. The hormones used to elicit these responses are indicated by name. Adapted from references 29, 43, 80.

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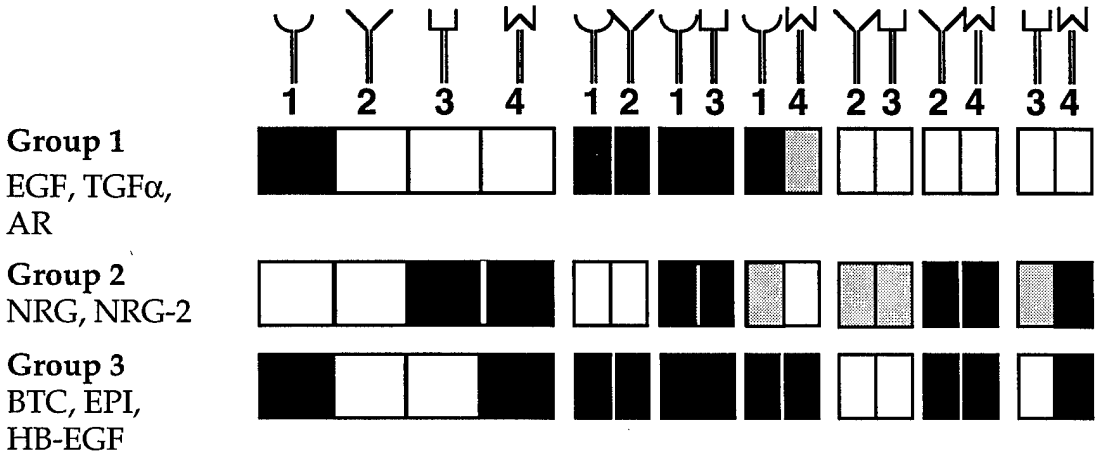
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Group 1
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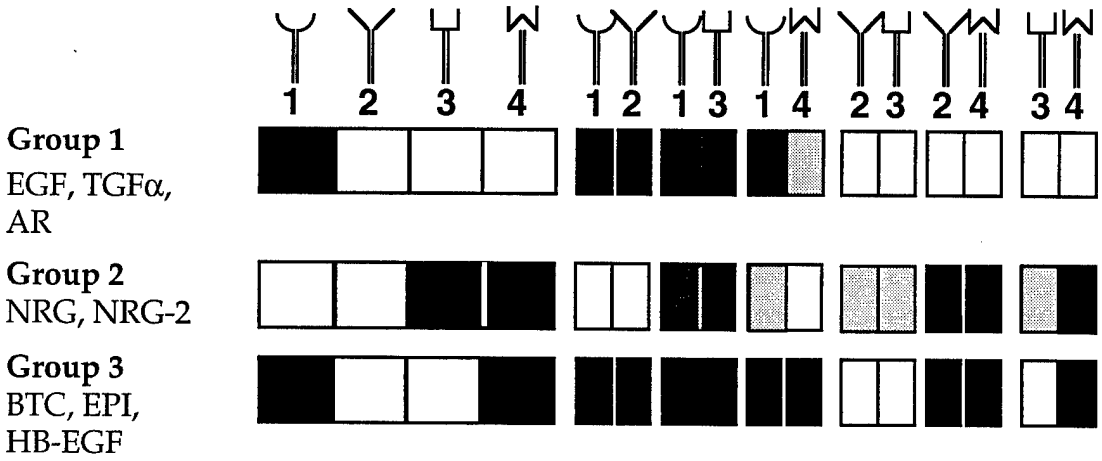
Group 2
NRG, NRG-2
Group 3
BTC, EPI, HB-EGF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